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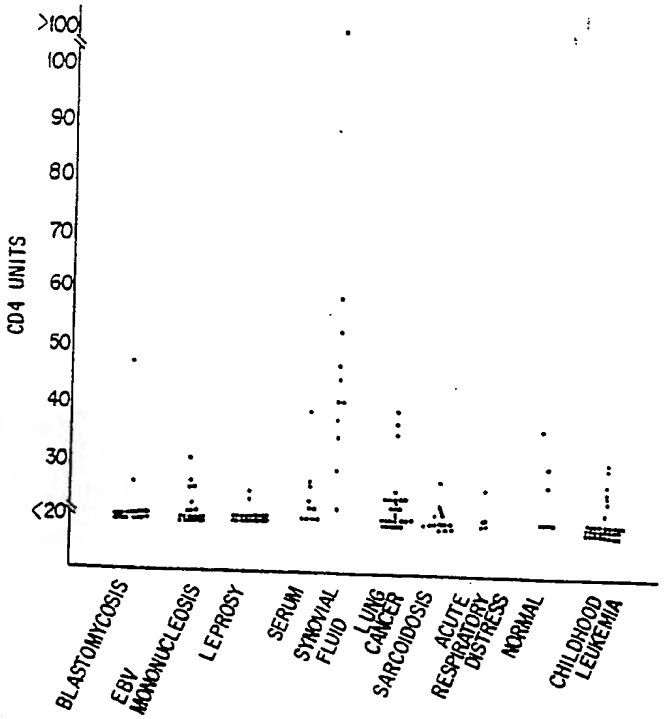
(54) Title: THERAPEUTIC AND DIAGNOSTIC METHODS USING SOLUBLE T CELL SURFACE MOLECULES

(57) Abstract

The present invention is directed to the measurement of soluble T cell growth factor receptors, soluble T cell differentiation antigens, or related soluble molecules or fragments thereof, and the use of such measurements in the diagnosis and therapy of diseases and disorders. Specific embodiments involve the diagnosis and monitoring of therapy using absolute values of such soluble molecules. Further embodiments involve detecting a change in the levels of such soluble molecules, in the diagnosis and therapy of diseases and disorders. In specific embodiments, detection of increases in both soluble IL2R and creatinine in the body fluid of a transplant patient can be used to differentially diagnose renal allograft rejection from infection. The invention is also directed to methods for measurement of soluble CD4 antigens, which measurements can be used, in a specific embodiment, to diagnose a state of immune activation, to diagnose rheumatoid arthritis, or to stage adult T cell leukemia in a patient. In another embodiment, soluble CD4 levels can be used to monitor the efficacy of disease treatments, a specific embodiment including monitoring therapy of HIV-infected patients. In another aspect, the invention relates to the detection, staging, or monitoring of therapy of diseases and disorders by measuring a plurality of soluble T cell markers.



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THERAPEUTIC AND DIAGNOSTIC METHODS USING SOLUBLE T CELL SURFACE MOLECULES

1. INTRODUCTION

The present invention is directed to the measurement of soluble T cell surface molecules, such as soluble T cell growth factor receptors and T cell differentiation antigens or fragments thereof, and the application of such measurements in the diagnosis and therapy of diseases and disorders. The measurement of such molecules, and preferably a plurality of such molecules, can be used in monitoring the effect of a therapeutic treatment, detecting and/or staging disease or in differential diagnosis of a physiological condition.

2. BACKGROUND OF THE INVENTION

2.1. T CELL GROWTH FACTORS AND RECEPTORS

T cells secrete a variety of polypeptides affecting immunoregulation of hematopoietic cells and are themselves subject to regulation by hormone peptides interacting with specific receptors on their cell surface. Interleukin 2 (IL-2), originally termed T cell growth factor, is synthesized and secreted by antigen- or lectin-activated T lymphocytes in the presence of macrophage-derived interleukin-1 and must interact with specific high-affinity membrane receptors to exert its biological effects (Smith, K.A., 1980, Immunol. Rev. 51:337-357; Leonard, W.J., et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:6957-6961). The interleukin 2 receptor (IL2R, Tac antigen) is not present on the surface of resting T or B lymphocytes. Upon activation by specific antigens or mitogens, T cell proliferation is mediated by an autocrine mechanism whereby activated cells secrete IL-2 and also express cell surface receptors for IL-2 (IL2R) (Leonard, W.J., et al., 1982, Nature 300:267; Meuer, S.C., et al., 1984, Proc. Natl. Acad. Sci. U.S.A.

81:1509). In addition to T cells, B cells (Mingari, M.C., et al., 1984, Nature 312:641-3; Pike, B.L., et al., 1984, Proc. Natl. Acad. Sci. U.S.A. 81:7917-21; Saiki, O., et al., 1988, J. Immunol. 140:853-8), NK cells (Ortaldo, J.R., et al., 1984, J. Immunol. 133:779-83; Kehrl, J.H., et al., 1988, J. Clin. Invest. 81:200-5) and possibly monocytes (Herrmann, F., et al., 1985, J. Immunol. 162:1111-6; Holter, W., et al., 1986, J. Immunol. 136:2171-75) express a membrane-bound IL2R.

Current evidence suggests that both chains of the IL-2 heterodimer receptor expressed on the surface of activated T cells, are encoded by a single gene on human chromosome 10 (Leonard, W.J., et al., 1984, Nature 311:626:31). The high affinity IL2R that functions to signal T cell cycle progression is composed of two distinct polypeptide chains, each of which contains an IL-2 binding site (Teshigawara, K., et al., 1987, J. Exp. Med. 165:223). The larger IL-2 binding protein (75 kD molecular weight) is designated as the beta chain, whereas the smaller protein (55 kD molecular weight) is termed the alpha chain (Smith, K.A., 1988, Adv. Immunol. 42:165-78). The alpha chain was the first IL-2 binding protein to become recognized as an "activation antigen" on the surface of activated T cells (hence the name anti-Tac for "T activated") (Uchiyama, T., et al., 1981, J. Immunol. 126:1393-7).

Interaction of IL-2 with its cell surface receptor results in a continuous T cell proliferation (Greene, W.C. and Leonard, W.J., 1986, Ann. Rev. Immunol. 4:69-95; Smith, K.A., 1984, Ann, Rev. Immunol. 2:319-333). Measurement of IL2R provides information on the state of immune activation of the lymphoid population. This has been accomplished by measuring IL2R on cell surfaces using flow cytometry or fluorescence microscopy. Using monoclonal antibodies which define the IL-2 receptor, altered IL-2 receptor expression has been reported in a number of immune abnormalities

(Greene and Leonard, supra; Depper, J.M., et al., 1984, J. Immunol. 133:1691-1695). Membrane IL2R has been found on certain B- or T-cell malignancies including Burkitt's lymphoma (Waldmann, T.A., et al., 1984, J. Exp. Med. 160:1450-1466), hairy cell leukemia (Waldmann et al., supra; Korsmeyer, S.J., et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:4522-4526), and human T cell leukemia virus (HTLV)-I-associated adult T cell leukemia (Depper, J.M., et al., 1984, J. Immunol. 133:1691-1695). The function of cellular IL2R in lymphoid malignancies has not been fully elucidated. Several cases of common, pre-B or T cell acute lymphoblastic leukemia (ALL) have been induced to express IL2R after in vitro activation (Touw, I., et al., 1985, Blood 66:556-561; Touw, I., et al., 1986, Blood 68:1088-1094; Matsuoka, M., et al., 1986, Leuk. Res. 10:597-603) and, in some cases, interleukin 2 stimulated subsequent colony formation of neoplastic progenitor cells in vitro (Touw, 1985, supra; Touw, 1986, supra).

Leukemia cells from some patients with T cell chronic lymphocytic leukemia were shown to have the receptors and a good proliferative response to exogenous interleukin 2 (Uchiyama, T., et al., 1985, J. Clin. Invest. 76:446-453; Tsudo, M., 1986, Blood 67:316-321). However, HTLV-1 associated adult T cell leukemia constitutively expressed high levels of cell surface IL2R but had no or very poor proliferative responses to interleukin 2 (Uchiyama, 1985, supra; Arya, S.K., et al., 1984, Science 223:1086-1087). Ebert et al. (1985, Clin. Immunol. Immunopathol. 37:283-297) have reported that T cells from patients with AIDS virus lack the ability to express IL2R on their surface even when the cell is activated.

Utilizing immunohistochemical staining, Kurnick reported high numbers of IL2R and HLA-DR positive cells in lung tumor infiltrating lymphocytes (Kurnick, J.T., et al., 1986, Clin. Immunol. Immunopath. 38:367-380).

2.2. T CELL SURFACE MOLECULES

Clusters of differentiation (CD) have been established which define human leukocyte differentiation antigens (Bernard and Boumsell, 1984, Hum. Immunol. 11:1-10), by the comparison of reactivities of monoclonal antibodies directed against the differentiation antigens. The T cell surface antigens, their classification into epitope-defined subgroups, and their distributions on T cells have been studied by use of monoclonal antibodies directed against human T cells (Clark et al., 1983, Immunogenetics 18:599-615; Hansen et al., 1984, in Leucocyte Typing, Bernard, A., et al., eds., Springer-Verlag, New York, pp. 195-212). Some of the T cell clusters of differentiation and other T cell surface molecules are listed in Table I.

TABLE I

T CELL SURFACE MARKERS

T Cell Surface Marker	Molecular Weight (kd)	Expression	Detection Monoclonal Antibody	Reference
T Cell Antigen Receptor	90	All T Cells	T40/25	Brenner, M.B., et al.,1984, J. Exp. Med. 160: 541-551
CD8	30/43	Suppressor/ cytotoxic (subset of T cells)	OKT8 Leu 2	Reinherz, E.L., et al., 1979, PNAS USA 76:4061-4065; Ledbetter, J.A., et al., 1981 Monoclonal Antibodies and T Cell Hybridoma Elsevier/ North Holland, N.Y., pp. 16-22.
T6	49	Thymocytes & Langerhans Cells Leukemia Cells	OKT6 NAI/34	Reinherz, 1979, supra.
CD4	62	Helper/Inducer Cells (subset of T cells)	OK4 Leu 3a	Kung, P.C., et al., 1979, Science 206: 347-349
CD3	19	Pan T Cell	OKT3	Kung, id.
TAC	50	IL-2 Receptor (Activated T Cells)	Anti-TAC	Uchiyawa, T., et al., 1981, J. Immunol. 126(4):1393-1397
T9	94	Transferrin Receptor (Activated T Cells)	OKT9	Reinherz, E.L., et al., 1980, PNAS USA 77:1588-1592
CD2	50	All T Cells	OKT11 Leu 5	Verbi, W., et al., 1982, Eur. J. Immunol. 12:81-86
VLA-1	130/165 /210	Late Activated T Cells	VLA-1	Helmer, M.E., et al., 1984, J. Immunol. 132:3011-3018

These T cell surface markers serve as markers of the cell lineage, the identity of the functional T cell subset to which the T cell belongs, and the activation state of the T cell. Several of the cell surface molecules have been studied in great detail and have been found to be important in initiating and regulating immune functions, and are critical to communication processes between immune cells. T cell antigen receptor, a surface molecule which comprises a disulfide-linker dimer of approximately 90 kilodaltons (kd), recognizes specific antigens and is responsible for initiating a complex series of biochemical events which constitute the T cell activation process (Meuer, S.C., et al., 1984, Ann. Rev. Immunol. 2:23-50; Acuto, O., et al., 1983, Cell 34:717-726). The CD3 structure is a three-chain complex associated with the T cell receptor (Kannellopoulos, J.M., et al., 1983, EMBO J. 2:1807; Borst, J., et al., 1983, Eur. J. Immunol 13:576; Van Den Elsen, P, et al., 1984, Nature 312:413; Meuer, S.C., et al., 1983, J. Exp. Med. 157:705). Lymphokine receptors, e.g. interleukin 2 (IL-2) receptor and interleukin 1 (IL-1) receptor, are essential for the activation and proliferation of T cells (Smith, K.A., 1984, Ann. Rev. Immunol. 2:319-333; Dower, S.K., et al., 1985, J. Exp. Med. 162:501-515). CD8 is a T cell specific surface glycoprotein expressed on the surface of approximately 30% of T lymphocytes associated with suppression and cytotoxic functions and the ability to recognize antigen in the context of class I MHC antigens (Swain, S.L., 1983, Immunol. Rev. 74:129-42). CD4 (OKT4 antigen) is a 55 kd glycoprotein expressed on the surface of approximately 60% of all T lymphocytes and is associated with helper function (Reinherz et al., 1979, Proc. Natl. Acad. Sci. U.S.A. 76:4061-4065) and the ability to recognize antigens in the context of type II MHC antigens (Swain,

supra; Meuer, S.C., et al., 1982, Proc. Natl. Acad. Sci. U.S.A. 79:4395-99). CD4 has also been identified as the receptor for the HTLV-III virus associated with acquired immune deficiency syndrome (AIDS) (McDougal, J.S., et al., 1986, Science 231:382-385). These various cell surface markers have enormous clinical application potentials for the identification of lymphocyte populations and their functional status (Krensky, A.M. and Clayberger, C., 1985, Transplant. 39(4):339-348; Kung, P.C., et al., 1984, Monoclonal Antibodies in Clinical Investigations, Clinical Biochemistry-Contemporary Theories and Techniques, vol. 3, Academic Press, pp. 89-115; Kung, P.C., et al., 1983, Int. J. Dermatol. 22(2):67-73).

Existing clinical methods of T cell typing involve the use of monoclonal antibodies which define T cell surface markers to detect the presence of specific cell surface markers on the T cell surface. Measuring the total numbers of T cells by surface markers has been useful for the characterization and classification of lymphoid malignancies (Greaves, M., et al., 1981, Int. J. Immunopharmac. 3(3):283-300). Changes in the relative percentage of T helper and T suppressor/cytotoxic cells were found to be associated with immune events in renal transplantation due to viral infection (Colvin, R.B, et al., 1981, Proc. 8th Int. Congr. Nephrol., Athens, pp. 990-996), autoimmune diseases (Veys, E.M., et al., 1981, Int. J. Immunopharmac. 3(3):313-319), and AIDS (Gupta, S., 1986, Clin. Immunol. Immunopathol. 38:93-100; Ebert, E.C., et al., 1985, Clin. Immunol. Immunopathol. 37:283-297).

The expression of T cell surface markers has also been used for the assessment of the immune status of patients. It has been established that by measuring the relative number of distinct, functional T cell subsets, and/or the relative number of activated T cells in peripheral blood or tissues, an assessment of the immunological condition of a

patient is possible. The activation antigens (e.g. IL-2 receptor) appear to be involved in T cell growth and differentiation processes.

Antibodies to CD4 have been widely described (Kung, P.C., et al., 1979, Science 206:347-349) and are commercially available. A series of such antibodies reacting with non-competing epitopes on the CD4 molecule have been described. Such a set has been termed OKT4, OKT4A, OKT4B, OKT4C, OKT4D, OKT4E, and OKT4F (Rao, P.E., et al., 1983, Cell. Immunol. 80:310).

Antibodies directed against the CD4 or CD8 antigens have been shown to block cell function. Antibodies against CD4 block most helper T functions, mixed lymphocyte reactions and induction of T helper activity (Biddison, W.E., et al., 1984, J. Exp. Med. 159:783). Antibodies against CD8 block the cytotoxic activity of CD8 positive cytotoxic T lymphocytes (Swain, S.L., 1981, Proc. Natl. Acad. Sci. U.S.A. 78:7101-7105). Antibodies against CD4 have also been described that are capable of activating CD4-positive T cells. CD4 is internalized upon treatment of the cells with phorbol esters and resulting phosphorylation (Hoxie, J.A., et al., 1986, J. Immunol. 137:1194-1201).

The cloning of the gene encoding CD4 reveals that it, like CD8, is a member of the immunoglobulin supergene family, containing both amino acid (32%) and structural (β sheets held together by disulfide bridges) homology at the V (variable)-like domain of CD4 to the V region of immunoglobulin (Maddon, P.J., et al., 1985, Cell 42:93-104). This V-like region of the molecule is followed by a stretch of 263 amino acids with no known homology to other molecules, followed by a transmembrane domain and highly charged cytoplasmic tail, containing serines which are phosphorylated upon activation (Littman, D.R., et al., 1984, Nature 325:453-55).

Homology between CD4 and CD8 is quite low. CD8 exists on the cell surface as dimeric or multimeric structures composed of a 33 kD monomer (Snow, P.M., et al., 1983, J. Biol. Chem. 258:14675-14681).

2.3. SOLUBLE IMMUNE CELL SURFACE MOLECULES

Several immune cell surface markers have been detected in the serum. The molecules of the human major histocompatibility complex (HLA molecules) are sets of cell surface glycoproteins involved in immune recognition. These macromolecular antigens have also been found to be present in body fluids such as serum (Pellegrino, M.A., et al., 1984, Meth. Enzymol. 108:614-624). The serum levels of Class I HLA-A and HLA-B have been shown to be present in sufficient quantity to perform HLA-typing in sera (Russo, C., et al., 1983, Transplant. Proc. 15(1):66-68; Pellegrino, M.A., et al., 1981, Transplant. Proc. 13(4):1935-1938). The presence of Class II HLA-DR in serum has also been detected (Sandrin, M.S., et al., 1981, J. Natl. Cancer Inst. 66(2):279-283; Russo, C., et al., 1983, Transplant. Proc. 15(1):57-59). The serum HLA-DR (Ia) has been shown to be markedly depressed in tumor patients.

A soluble form of IL2R has been detected (Rubin et al., 1985, J. Immunol. 135:3172-3177; Rubin et al., 1985, Fed. Proc. 44:946; U.S. Patent No. 4,707,443 by Nelson et al.) that is released by activated normal peripheral blood mononuclear cells and synthesized in large amounts in vitro by HTLV-I-infected leukemic cell lines. A sandwich enzyme immunoassay was used to quantitate the soluble IL2R.

Little is known about the functional significance of soluble IL2R. Since soluble IL2R is capable of binding interleukin 2 (Rubin, L.A., et al., 1985, J. Immunol. 135:3172-3177), it may have an immunoregulatory role by competing with cellular IL2R for the ligand and thus down-regulating the immune response. In this regard, the soluble

IL2R has been suggested to be a "blocking factor" produced by the malignant cells to inhibit the host's immune response to the tumor (id.).

Subsequent studies have disclosed comparable levels of soluble IL2R in cord blood and peripheral blood from normal adults (Nelson, D.L., et al., 1986, Pediatr. Res. 20:136-139). Increased serum levels of IL2R have been found in patients with certain B or T cell malignancies (Nelson, D.C., 1986, Fed. Proc. 45:377; Saadeh, C., et al., 1986, Fed. Proc. 45:378; MacKeen, L., et al., 1986, Fed. Proc. 45:454; Reuben, J.M., et al., 1986, Blood 68(5), Supp. 1:213a). Elevated levels of soluble IL2R have also been reported present in the serum of aged subjects (Saadeh, C., et al., 1986, Fed. Proc. 45:378), and in patients with AIDS (Saadeh, supra).

Several other cell surface markers which are primarily present on T cells have also been found in soluble form. CD2, a T cell surface molecule present in all normal T cells and a receptor for sheep red blood cells, has been detected at higher levels in the sera of certain cancer patients than those found in normal control patients (Falcao, R.P., et al., 1984, Clin. Lab. Immunol. 13:141-143; Oh. S.-K., et al., 1985, Scand. J. Immunol. 22:51-60). CD8 (Leu 2, OKT8), a surface marker found on the surface of suppressor/cytotoxic T cells and which may be involved in cellular recognition, has also been reported at highly elevated levels in the serum of patients with T cell leukemia (Fujimoto, J., et al., 1983, J. Exp. Med. 159:752-766). Leu-1, another T cell surface molecule, was measured in serum following anti-Leu-1 monoclonal antibody treatment (Miller, R.A., et al., 1982, New Engl. J. Med. 306:517-520). Oh et al. (1985, supra) reported that less than half of the patients with malignancies in their study presented elevated levels of soluble OKT11 receptor in their serum.

However, not all T cell surface molecules are released into the serum (Fujimoto, J., et al., 1983, J. Exp. Med. 159:752-766). Leu 1 antigen was not detectable in the serum of normal or leukemic patients who have not received antibody therapy. Leu 3 antigens were also not detectable in soluble form in T cell culture supernatants (id.).

Fujimoto et al. (1983, J. Exp. Med. 159:752-766) were unable to find evidence of released CD4 using a sandwich enzyme immunoassay based on Leu3b and Leu3a (Becton-Dickinson). This assay worked well with detergent lysates of cells but did not detect released CD4 in culture supernatants of $CD4^+$ and $CD8^+$ leukemic T cells which could be shown to release CD8, or in culture supernatants of $CD4^+$ T cells.

PCT Publication No. WO 87/03600, published June 18, 1987, entitled "Assay Systems for Detecting Cell Free T Cell Antigen Receptor Related Molecules and Clinical Utilities of the Assays" concerns methods for diagnosing diseases and for monitoring diseased conditions by measuring the amount of soluble T cell antigen receptor in a subject's body fluid.

PCT Publication No. WO 87/05912, published October 8, 1987, entitled "Therapeutic and Diagnostic Methods Using Soluble T Cell Surface Molecules" relates to the measurement of certain soluble T cell growth factor receptors and soluble T cell differentiation antigens in the diagnosis and therapy of various diseases and disorders.

3. SUMMARY OF THE INVENTION

The present invention is directed to the measurement of soluble T cell growth factor receptors, soluble T cell differentiation antigens, or related soluble molecules or fragments thereof, and the use of such measurements in the diagnosis and therapy of diseases and disorders. The measurement of such molecules can be valuable in monitoring the effect of a therapeutic treatment on a subject,

detecting and/or staging a disease in a subject, and in differential diagnosis of a physiological condition in a subject. These measurements can also aid in predicting therapeutic outcome and in evaluating and monitoring the immune status of patients.

In a specific embodiment, a rise in both soluble IL2R and creatinine in a body fluid of a patient can be used to predict allograft rejection or to differentially diagnose renal allograft rejection from infection in a transplant patient. In another particular embodiment, a change in serum IL2R concentrations in serial samples can be more sensitive than the absolute level of serum IL2R for the diagnosis of rejection.

The invention is also directed to the measurement of serum IL2R levels to stage non-lymphatic malignancies.

The invention also relates to immunoassays which preferentially detect soluble CD4 over the cell-surface CD4.

An increase in soluble CD4 antigen levels in a sample from a patient can be used to diagnose a state of immune activation. Such an increase in soluble CD4 antigen levels in synovial fluid can be used to diagnose rheumatoid arthritis. Soluble CD4 measurements can also be used to stage adult T cell leukemia, or determine the phenotype of a cell in culture. Soluble CD4 measurements can also be used to monitor AIDS patients undergoing therapy.

The invention also relates to the measurement of a plurality of soluble T cell surface markers, for the detecting, staging, or monitoring of treatment of a disease or disorder. In particular embodiments, the measurement of a plurality of soluble T cell surface markers and their change relative to one another can be superior to the

measurement of any soluble T cell surface marker alone, for the detecting, staging, or monitoring of treatment of a disease or disorder.

In particular embodiments, measurements of the soluble T cell surface molecules can be accomplished by sandwich enzyme immunoassays. Kits for such measurements are also provided.

3.1. DEFINITIONS

As used herein, the following abbreviations will have the meanings indicated:

Staging a disease	=	assessing the degree of severity according to standard classifications
AC	=	adenocarcinoma
CsA	=	cyclosporin A
HC	=	hairy cell leukemia
HTLV III/LAV/HIV	=	Human T Cell Leukemia Virus Type I/Lymphadenopathy Associated Virus/Human Immunodeficiency Virus
IL-1	=	interleukin-1
IL-2	=	interleukin-2
IL2R	=	interleukin-2 receptor
mAb	=	monoclonal antibody
PBMC	=	peripheral blood mononuclear cell
PHA	=	phytohemagglutinin
SCLC	=	squamous cell lung carcinoma
Spontaneous release	=	release by normal or pathologic physiological processes of the cell
AZT	=	azido-deoxythymidine
RF	=	rheumatoid factor

4. DESCRIPTION OF THE FIGURES

Figure 1. Levels of soluble CD4 in sera of normal individuals and patients from a number of disease groups. The assay used was as described in Section 6.2.1, infra. CD4 antigen was detected using mAb 8F4 as capture reagent and mAb R2B7 as detection reagent in a sandwich immunoassay. The limit of sensitivity for the assay was 20 units.

Figure 2. Levels of soluble CD4 in sera of normal individuals and patients from a number of disease groups. The assay used was as described in Section 6.2.2, infra. SF: synovial fluid; EBV/mono: Epstein Barr Virus/mononucleosis.

Figure 3. Longitudinal studies of soluble CD8 levels in sera of patients with Kaposi's sarcoma (KS) or with AIDS-related complex (ARC). Closed diamonds: soluble CD8 levels (U/ml); Open diamonds: HIV p24 levels (pg/ml) x 10.

Figure 4. Serial serum IL2R (units/ml) and creatinine (Cr) (mg/dl) levels in a renal allograft recipient who developed a rejection episode that responded to OKT3 antibody therapy (arrow marks day of biopsy diagnosis). The rise in serum IL2R accompanied and preceded the rise in serum creatinine.

Figure 5. Dot plots of the change in serum IL2R (A), urine IL2R (B), or serum creatinine (Cr) (C) concentrations by clinical status: stable (S), rejection (R), cyclosporine toxicity (C), and infection (I). Samples of rejection are from 2 days before the first rise in Cr to the time of institution of antirejection therapy.

Figure 6. A plot of the sensitivity and specificity of the serum IL2R assay and serum creatinine (Cr) for the diagnosis of rejection. The data from Figure 5(a,c) are given in the form of receiver operating characteristic (ROC) curves. In this display, the threshold for a positive test is varied over a broad range (here from the 70th to the 99th percentile of stable patients). The further the curves are

from the diagonal line (which represents chance alone), the better the discrimination of the test. The curves for IL2R and Cr are equivalent.

5. DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to the measurement of soluble T cell growth factor receptors, soluble T cell differentiation antigens, or related soluble molecules or fragments thereof, and the use of such measurements in the diagnosis and therapy of diseases and disorders.

As used herein, the term "soluble" shall mean those molecules that are "spontaneously released"; i.e., released by normal or pathologic physiological processes of the cell. Such molecules are to be distinguished from "solubilized" cell surface forms of the molecules, whose solubilization is brought about by in vitro manipulation such as cell lysis by detergent. The soluble T cell markers (antigens and receptors) of the invention are molecules which carry antigenic determinants of their cell-surface counterparts.

Proteinaceous molecules, or fragments thereof, derived from the surface of T cells, and proteinaceous molecules which have immunologically similar counterparts present on the surface of T cells or activated T cells, which are present in a body fluid and not associated with the surface of a T cell are soluble T cell surface molecules of the invention. These molecules can be either glycosylated or nonglycosylated and may be soluble by themselves or considered soluble by virtue of their association with other soluble molecules.

The measurement of the soluble molecules of the invention can be valuable in monitoring the effect of a therapeutic treatment on a subject, detecting and/or staging a disease in a subject, and in differential diagnosis of a physiological condition in a subject. These measurements can also aid in predicting therapeutic outcome and in

evaluating and monitoring the immune status of patients. More than one type of soluble molecule can be measured. The soluble molecules can be measured in any body fluid of the subject including but not limited to serum, plasma, urine, and saliva.

5.1. MONITORING THE EFFECT OF A THERAPEUTIC TREATMENT

The present invention provides a method for monitoring the effect of a therapeutic treatment on a subject who has undergone the therapeutic treatment. This method comprises measuring at suitable time intervals the amount of a soluble molecule or soluble fragment thereof which comprises, or is immunologically related to, a T cell growth factor receptor or T cell differentiation antigen. Any change or absence of change in the amount of the soluble molecule can be identified and correlated with the effect of the therapeutic treatment on the subject. In a specific embodiment of the invention, soluble molecules immunologically related to the CD4 antigen can be measured in the serum of patients by a sandwich enzyme immunoassay in order to evaluate the therapeutic efficacy of, for example, administration of immunomodulators such as alpha-interferon, Cyclosporin A, and monoclonal antibody OKT3. In another embodiment of the invention, the levels of soluble CD4 molecules can be measured in the serum of AIDS patients in order to evaluate the therapeutic efficacy of treatments such as administration of AZT, interferon or CD4 itself.

The therapeutic treatments which may be evaluated according to the present invention include but are not limited to radiotherapy, drug administration, vaccine administration immunosuppressive or immunoenhancive regimens, etc. The immunosuppressant regimens include, but are not limited to administration of drugs such as Cyclosporin A, chlorambucil, cyclophosphamide, or azathioprine, and anti-T cell antibody such as anti-T3

monoclonal antibody and anti-thymocyte globulin, etc. The immunoenhancive regimens include, but are not limited to administration of interleukin-1, interleukin-2, and other T cell growth factors.

5.2. DETECTING AND/OR STAGING A DISEASE IN A SUBJECT

In another embodiment of the present invention, measurement of a soluble molecule which comprises, or is immunologically related to, a T cell growth factor receptor or T cell differentiation antigen can be used to detect and/or stage a disease or disorder in a subject. The measured amount of the soluble molecule is compared to a baseline level. This baseline level can be the amount which is established to be normally present in the body fluid of subjects with various degrees of the disease or disorder. An amount present in the body fluid of the subject which is similar to a standard amount, established to be normally present in the body fluid of the subject during a specific stage of the disease or disorder, is indicative of the stage of the disease in the subject. The baseline level could also be the level present in the subject prior to the onset of disease or the amount present during remission of disease. Diseases or disorders which may be detected and/or staged in a subject according to the present invention include but are not limited to those listed in Table II, infra.

TABLE II

DISEASES AND DISORDERS WHICH MAY BE DETECTED AND/OR STAGED IN A SUBJECT ACCORDING TO THE PRESENT INVENTION

I. Infectious Diseases
- Induced by virus:
  - Herpesvirus
  - Cytomegalovirus
  - Epstein-Barr Virus
  - HTLV-I
  - HTLV-III/LAV/HIV (AIDS)

II. Cancer
- T cell leukemia
  - HTLV-I-associated adult T cell leukemia
- T cell lymphoma
- Burkitt's lymphoma
- Hairy cell leukemia
- Sezary syndrome
- Hodgkin's disease
- Chronic lymphocytic leukemia
- Non-Hodgkin's lymphoma
- B-cell acute lymphoblastic leukemia
- Solid tumors

III. Autoimmune Diseases
- Rheumatoid arthritis
- Diabetes
- Multiple sclerosis
- Systemic lupus erythematosis

IV. Organ Allograft Rejection

In specific embodiments of this aspect of the invention, measurements of plasma or serum levels of the soluble molecules can be used in the detection of disease, or to determine disease stage and assign risk.

Rheumatoid arthritis can be monitored by measuring soluble CD4 levels in a patient. In a preferred embodiment, elevated levels of soluble CD4 in synovial fluid relative to serum is a diagnostic indication of rheumatoid arthritis.

In another embodiment of the invention, detection of an increase in soluble CD4 antigen in the body fluid of a patient can be used to diagnose a state of immune activation. Soluble CD4 measurements can also be used to detect and/or stage adult T cell leukemia. Elevation of CD4 antigen levels in the synovial fluid of a patient can indicate rheumatoid arthritis. In yet another embodiment, the detection of soluble CD4 in cell culture supernatants can be relied on as an indication of the $CD4^+$ phenotype of the lymphocytes present.

5.3. DIFFERENTIAL DIAGNOSIS OF A PHYSIOLOGICAL CONDITION

In another embodiment of the invention, the measurement of soluble T cell growth factor receptors, T cell surface antigens, or immunologically related molecules can be used to differentially diagnose in a subject a particular physiological condition as distinct from among two or more physiological conditions. To this end, the measured amount of the soluble molecule is compared with the amount of the soluble molecule normally present in a body fluid of a subject with one of the suspected physiological conditions. A measured amount of the soluble molecule similar to to the amount normally present in a subject with one of the physiological conditions, and not normally present in a subject with one or more of the other physiological conditions, is indicative of the physiological condition of the subject.

In a specific embodiment of this aspect of the invention, measurement of soluble molecules can be used in the differential diagnosis of renal allograft rejection, especially in distinguishing Cyclosporin A nephrotoxicity or infection. Similar differential diagnosis of allograft rejection using the methods of the invention can be applied to other organ allografts, including but not limited to liver, heart, and pancreas.

In a preferred embodiment of the invention, the measurement of changes in the levels of soluble molecules, rather than measurement of the absolute levels of the soluble markers, can be used to differentially diagnose renal allograft rejection.

5.4. SOLUBLE T CELL GROWTH FACTOR RECEPTORS, T CELL DIFFERENTIATION ANTIGENS, AND RELATED MOLECULES

Any T cell surface molecule or immunologically related molecule which is present in soluble form in the body fluid at levels which correlate with a disease condition or disorder, or a stage thereof, may be used in the practice of the present invention. T cell surface markers which may potentially be used include but are not limited to those listed in Table I, supra.

In specific embodiments, the soluble form of the CD4, CD8, and IL2R molecules may be measured.

5.4.1. KITS AND ASSAYS FOR MEASUREMENT

Any procedures known in the art for the measurement of soluble molecules can be used in the practice of the instant invention. Such procedures include but are not limited to competitive and non-competitive assay systems using techniques such as radioimmunoassays, ELISA (enzyme linked immunosorbent assay), "sandwich" immunoassays, precipitin reactions, gel diffusion precipitin reactions, immunodiffusion assays, agglutination assays, complement-

fixation assays, immunoradiometric assays, fluorescent immunoassays, protein A immunoassays, and immunoelectrophoresis assays, to name but a few.

In a preferred embodiment, a sandwich enzyme immunoassay can be used. One description of such an embodiment follows: A monoclonal antibody (capture antibody, mAb 1) directed against the soluble antigen is adsorbed onto a solid substratum. The soluble antigen present in the sample binds to the antibody, and unreacted sample components are removed by washing. An enzyme-conjugated monoclonal antibody (detection antibody, mAb 2) directed against a second epitope of the antigen binds to the antigen captured by mAb 1 and completes the sandwich. After removal of unbound mAb 2 by washing, a substrate solution is added to the wells. A colored product is formed in proportion to the amount of antigen present in the sample. The reaction is terminated by addition of stop solution and absorbance is measured spectrophotometrically. A standard curve is prepared from known concentrations of the soluble antigen, from which unknown sample values can be determined. In particular embodiments, such an assay may be used to determine soluble IL2R levels or soluble T cell antigen levels. In a preferred embodiment for the measurement of IL2R levels, anti-IL2R mAbs 2R12 and 7G7 can be used as the capture and detection antibodies, respectively, in a sandwich immunoassay (such as the CELLFREE® IL2R Test Kit assay, T Cell Sciences, Cambridge, MA). In a preferred embodiment for the measurement of soluble CD8 antigen levels, anti-CD8 mAbs 4C9 and 5F4 can be used as the capture and detection antibodies, respectively, in a sandwich enzyme immunoassay (such as the CELLFREE® T8 Test Kit assay, T Cell Sciences, Cambridge, MA). In a preferred embodiment for the measurement soluble CD4 antigen

levels, anti-CD4 mAbs 8F4 and R2B7 can be used as the capture and detection reagents, respectively, in a sandwich enzyme immunoassay (see Sections 5.4.3 and 6, infra).

Kits for carrying out the assays of, and for use in the practice of, the present invention are also within the scope of the invention. For instance, such a kit can comprise a pair of antibodies to the same T cell marker (receptor or antigen), which antibodies do not compete for the same binding site on the marker. In another embodiment, a kit can comprise more than one pair of such antibodies, each pair directed against a different T cell marker, thus useful for the detection or measurement of a plurality of T cell markers.

5.4.2. FORMULATION OF AN IMMUNOASSAY FOR THE PREFERENTIAL DETECTION OF SOLUBLE FORMS OF T CELL SURFACE MARKERS OVER SOLUBILIZED FORMS

The present invention also provides a way of deriving immunoassay systems which preferentially detect/quantitate physiologically released (soluble) forms of cell surface markers over solubilized (e.g., detergent-treated) cell surface markers. Such a method involves the use of recombinant forms of the specific cell surface marker to be assayed, which have been genetically engineered to be physiologically soluble (i.e., by deletion of DNA sequences encoding the transmembrane region). Such recombinant forms are likely to lack epitopes found on the transmembrane region, which epitopes are thus specific to the solubilized cell surface marker and which epitopes are likely also to be absent from the physiologically released form of the marker. Thus, the recombinant molecule can be used to screen anti-cell surface marker antibodies for determination of the appropriate antibodies to be used for preferential detection of the physiologically released form of the surface marker. Pairs of antibodies can be screened for optimization of a

sandwich ELISA for detection of soluble cell-surface marker. This aspect of the invention is illustrated by way of example in Section 6, infra, where a soluble CD4 assay is devised that preferentially detects soluble CD4 relative to solubilized CD4.

Antibodies can be produced for testing for suitability for use in the detection of soluble forms of T cell surface markers. Such antibodies can be polyclonal or monoclonal. Monoclonal antibodies are preferred for use.

Various procedures known in the art may be used for the production of polyclonal antibodies to epitopes of a given T cell surface molecule. For the production of antibody, various host animals can be immunized by injection with a T cell surface molecule, a recombinant version thereof, synthetic protein, or fragment thereof, including but not limited to rabbits, mice, rats, etc. In a preferred embodiment, the immunogen is a truncated recombinant soluble form of the T cell surface molecule. Various adjuvants may be used to increase the immunological response, depending on the host species, and including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and corynebacterium parvum.

A monoclonal antibody to an epitope of the T cell surface molecule can be prepared by using any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include but are not limited to the hybridoma technique originally described by Kohler and Milstein (1975, Nature 256:495-497), and the more recent human B cell hybridoma technique (Kozbor et al.,

1983, Immunology Today 4:72) and EBV-hybridoma technique (Cole et al., 1985, Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96).

In one embodiment, the monoclonal antibodies may be human monoclonal antibodies or chimeric human-mouse (or other species) monoclonal antibodies. Human monoclonal antibodies may be made by any of numerous techniques known in the art (e.g., Teng et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:7308-7312; Kozbor et al., 1983, Immunology Today 4:72-79; Olsson et al., 1982, Meth. Enzymol. 92:3-16). Chimeric antibody molecules may be prepared containing a mouse (or rat, or other species) antigen-binding domain with human constant regions (Morrison et al., 1984, Proc. Natl. Acad. Sci. U.S.A. 81:6851; Takeda et al., 1985, Nature 314:452).

A molecular clone of an antibody to an epitope of a T cell surface molecule can be prepared by known techniques. Recombinant DNA methodology (see e.g., Maniatis et al., 1982, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York) may be used to construct nucleic acid sequences which encode a monoclonal antibody molecule, or antigen binding region thereof.

Antibody molecules may be purified by known techniques, e.g., immunoabsorption or immunoaffinity chromatography, chromatographic methods such as HPLC (high performance liquid chromatography), or a combination thereof, etc.

Antibody fragments which contain the idiotype of the molecule can be generated by known techniques. For example, such fragments include but are not limited to: the $F(ab')_2$ fragment which can be produced by pepsin digestion of the antibody molecule; the Fab' fragments which can be generated

by reducing the disulfide bridges of the $F(ab')_2$ fragment, and the Fab fragments which can be generated by treating the antibody molecule with papain and a reducing agent.

Once specific antibodies are demonstrated to be suitable for use in the preferential detection of soluble T cell-surface molecules, other such suitable antibodies may be selected by virtue of their having the same epitope specificity as the former antibodies. Such similar epitope specificity can be ascertained, for example, by observing the ability of a second antibody to inhibit binding of a first antibody to its antigen.

5.4.3. SOLUBLE CD4 AND ASSAY FOR ITS DETECTION

The invention is also directed to assays for measurement of soluble (released) CD4, which assays preferentially measure soluble CD4 over the solubilized membrane form of CD4. Examples of such assays are detailed infra in Section 6.

In a preferred embodiment for the detection or measurement of soluble CD4 antigen levels, anti-CD4 mAbs 8F4 and R2B7 can be used as the capture and detection reagents, respectively, in a sandwich immunoassay.

We also demonstrate infra that the physiologically released form of CD4 is physically different from that of the solubilized cell surface form, and that assays which quantitate the solubilized cell surface form do not necessarily quantitate the released form.

Soluble CD4 has been specifically quantitated, according to the present invention, and has been shown to be a reliable indicator of various pathologic conditions (see Section 6, infra). Thus, detection and/or measurement of soluble CD4 can be used to diagnose, to monitor, and/or to stage various diseases and disorders involving the immune system.

5.4.4. DETECTING OR STAGING OF DISEASE OR MONITORING OF RESPONSE TO TREATMENT IN PATIENTS BY MEASUREMENT OF A PLURALITY OF SOLUBLE T CELL SURFACE MARKERS

The present invention also provides for the detecting or staging of disease, or the monitoring of treatment by measuring a plurality (at least two) T cell surface markers (receptors or differentiation antigens). For example, a plurality of soluble T cell markers selected from among soluble IL2R, CD4, and CD8 can be measured to diagnose, stage, or monitor treatment of diseases or disorders. Such diseases or disorders include those discussed supra in Sections 5.1 through 5.3 (e.g., see Table II). Soluble marker levels can represent a measure of immune system function, paralleling disease course or treatment efficacy. In a preferred embodiment, the prognostic indicator is the observed change in different marker levels relative to one another, rather than the absolute levels of the soluble markers present at any one time. Since soluble CD4, soluble CD8 and soluble IL2R levels are measures of the immune system itself, they should provide a much improved measure of the relative health of the immune system during various stages of diseases or disorders.

In a preferred embodiment, measurements of a plurality of soluble T cell surface markers are used to detect, stage, or monitor treatment of diseases and disorders caused by HIV (the causative agent of AIDS) infection.

Since the discovery of AIDS and the observation that the AIDS virus, HIV, binds to the T4, or CD4, receptor, there have been several proposals for the treatment of AIDS patients or for the development of vaccines for populations of people at risk. These include the treatment of AIDS patients with drugs such as AZT (azido-deoxythymidine), γ or β interferons, and with soluble CD4, or its fragments and derivatives, and the production of potential AIDS vaccines, such as gp120 peptides. What is very much needed is a procedure that can be used to monitor the efficacy of these

treatments or vaccines. To date, the levels of the HIV antigen p24 have not proved sensitive enough. With the observation herein described that soluble CD4 in particular, but soluble CD8 and soluble IL2R receptors as well, can be identified and detected in HIV-infected patients with different manifestations of disease, it becomes possible to develop a sensitive immunoassay to monitor AIDS therapies and vaccines. The CELLFREE® Test Kit (T Cell Sciences, Cambridge, MA) assays can be useful for this purpose. Due to the intimate involvement of CD4 in the etiology of AIDS, it is expected that spontaneously released soluble CD4 levels should be extremely sensitive markers of the state of immune function during various stages of HIV infection and therapeutic treatments. This is especially true, as soluble CD4 is produced when $CD4^+$ cells become activated, (see Section 6.2, supra) as occurs during HIV infection. Furthermore, measurements of other soluble T cell markers, such as soluble IL2R and soluble CD8, that also indicate the state of immune function should be valuable.

The best index for monitoring AIDS treatment or disease progression can be a profile of soluble T cell markers, such as soluble CD4, CD8 and IL2R, rather than any individual marker alone (see Sections 6.3 and Section 7). Such a profile can be obtained by determining the soluble receptor levels of a panel of soluble receptors in longitudinal samples of sera from patients undergoing treatment.

In a preferred aspect, the approach that can be taken is to determine the levels of soluble CD4 (and soluble CD8 and soluble IL2R) levels in longitudinal time studies and to compare these values with a baseline level. The baseline level can be either the level of the soluble marker present in normal, disease free individuals; and/or the levels

present prior to treatment, or during remission of disease, or during periods of stability. These levels can then be correlated with the disease course or treatment outcome.

6. SOLUBLE CD4 ANTIGEN

In the examples detailed herein, a sandwich immunoassay is described for the preferential detection of the soluble form of CD4 antigen relative to the cell-surface form of CD4 antigen.

6.1. MATERIALS AND METHODS

6.1.1. ANTIBODIES

Antibody Leu3a, biotin or FITC labeled, was purchased from Becton Dickinson, Mountain View, California. Antibodies OKT4 and OKT4A were obtained from Ortho Diagnostics, Raritan, New Jersey. Antibody IOT4 was obtained from Immunotech, Cedex, France and further purified by ammonium sulfate precipitation. Antibodies B67.2 and B66.1 were from G. Trincherie, Wistar Institute. Antibody 3G2 was from Sanchex Madrid, Madrid, Spain. Antibody R2B7 was obtained from a fusion of rat spleen cells (carried out according to standard procedures), from an animal immunized with whole human peripheral blood lymphocytes, with mouse SP2/0 myeloma cells. Clone R2B7 was selected from this fusion based on its ability to stain populations of peripheral blood lymphocytes identical to these identified by OKT4.

Antibodies were purified either by ammonium sulfate precipitation or by protein A sepharose using the Biorad MPAS buffer system (BioRad Corporation, Richmond, California). Horseradish peroxidase (HRP) conjugates were prepared essentially according to the method of Wilson and

Nakane (1978, in Immunofluorescence and Related Techniques, Knapp, W., et al., eds. Elsevier, p. 215) using a molar HRP to antibody ratio of four.

Antibodies generated from a fusion of mice immunized with intact T cells (Jurkat) were screened for their ability to substitute for Leu3A in the assay as follows: Plates were coated with R2B7 as described, blocked and incubated with recombinant soluble CD4 for 2 hours at 37°C. Plates were washed and 50 µl of each hybridoma supernatant at 1-10 µg/ml were added followed by 50 µl of biotinyl Leu3A. Following a 2 hour incubation, plates were washed and 100 µl of streptavidin peroxidase (0.5 µg/ml) was added for 30 minutes. Plates were washed and developed as described below.

6.1.2. IMMUNOASSAY PROTOCOLS

6.1.2.1. INITIAL ASSAY

The enzyme immunoassay for the CD4 antigen was based on the sandwich immunoassay technique. Briefly, each well of a microtiter plate (Nunc, certified high binding) was coated overnight at 4°C with a solution of murine monoclonal anti-human CD4 antibody in PBS, pH 7.4. Any remaining protein-binding sites on the microtiter wells were then blocked for two hours at 37°C with 300 µl per well of a solution of BSA (1%) (Kirkegard and Perry Laboratories, Maryland) and Tween 20 (0.05%) (Zymed Laboratories, South San Francisco, California) in phosphate buffered saline (PBS), pH 7.4. The wells were then washed three times with 350 µl per well of PBS (pH 7.4) with 0.05% Tween 20. Following the final wash step, the wash solution was aspirated from the wells and 50 µl of a sample diluent consisting of 0.15 M NaCl, 25 mM Tris (pH 7.4) supplemented with bovine proteins was added to each well. Fifty µl of standard or sample were added to the appropriate wells in duplicate. The solution in the wells was mixed thoroughly

by gently tapping the side of the plate for fifteen seconds. The plate was then sealed and incubated at 37°C for 2 hours. At the end of this incubation period, the solution was aspirated from the plate and each well was washed three times with 350 µl of PBS/Tween 20 as above. One hundred µl of horseradish peroxidase (HRP) conjugated murine monoclonal anti-human CD4 antibody was added to each well of the microtiter plate, and the plate was again incubated at 37°C for 2 hours, as above. At the end of this incubation, the wells were once again washed three times with PBS/Tween 20 as above. One hundred µl of o-phenylenediamine (0.2%) dissolved in 0.1 M sodium citrate buffer, pH 5.5, was then added to each well of the plate and incubated at 24°C ± 2°C for 30 minutes. At the end of this final incubation, 50 µl of 2 N H_2SO_4 was added to each well to stop the reaction and the absorbance of each well was read at 490 nm.

For assays involving biotinylated antibodies, biotin conjugates were substituted for the HRP antibody conjugate. After the 2 hour incubation, wells were washed and 100 µl of streptavidin horseradish peroxidase (Zymed Laboratories) at 0.5 µg/ml in 1% bovine serum albumin in tris buffered saline was added. Following a 30 minute incubation at 37°C, wells were washed and color developed as described above.

Where indicated, assays were also performed as a single step assay in which conjugated antibody was added at the same time as the sample and incubated for 4 hours at room temperature on a rotating shaker platform, after which washing and color development were performed as described.

6.1.2.2. OPTIMIZED ASSAY

The configuration of the initial assay was modified by optimizing each of the assay reagents. This resulted in an improved sensitivity for the overall assay where much lower

levels of soluble CD4 could be reliably and reproducibly detected. The optimized assay configuration is given in Table III.

TABLE III

COMPARISON OF INITIAL & OPTIMIZED ASSAY CONFIGURATIONS

INITIAL ASSAY	OPTIMIZED ASSAY
Blocking Buffer:	
1% BSA + 0.05% Tween 20	0.5% Casein, 0.008% NP-40, 0.005% EDTA
Sample Diluent*	
0.15 M NaCl, 25mM Tris, supplemented with bovine proteins	0.25% NP-40, supplemented with bovine proteins in PBS
Conjugate Diluent*	
25% FCS in Tris buffered saline + 0.25% NP-40	15% FCS, 0.15% NP-40

* Aggregated IgG was added to both the sample diluent and the conjugate diluent to remove any effect of rheumatoid factors in various samples.

Occasionally, it was observed that the presence of rheumatoid factor (RF) in some of the samples led to erroneous determinations of soluble CD4 that appeared as false positives. To remove this effect, aggregated IgG was added to the sample and conjugate diluent buffers. The aggregated IgG was prepared by heating a 100 μg/ml solution of IgG in 100 mM sodium phosphate buffer, 0.9% NaCl, pH 5.56 at 56-60°C for 50 minutes, followed by neutralization with dibasic sodium phosphate, 0.9% NaCl, pH 8 to give a final pH of 7.4.

6.1.3. CELL PROCEDURES

For stimulation experiments, peripheral blood mononuclear cells were prepared using Ficoll Hypaque gradients. Cells were put into culture along with phytohemagglutinin (PHA) (0.5 μg/ml) or phorbol myristate acetate (1 ng/ml) and ionophore A2317 (0.1 ng/ml) or OKT3 (anti-T3 monoclonal antibody) (2 μg/ml). Samples were taken daily. For long term cultures of cells from rheumatoid arthritis or lung cancer patients, cells were maintained on IL-2. Cells were removed from culture supernatants by centrifugation followed by filtration through 0.22 μm filters and stored frozen until analysis.

Cell surface phenotyping was performed using a Cytofluorograph II (Ortho Diagnostic System, Westwood, Massachusetts) and FITC labeled OKT4 or OKT8 (Ortho Diagnostic Systems, Raritan, New Jersey).

Recombinant souble CD4 was obtained from cell culture supernatant of a chinese hamster ovary (CHO) cell line transfected with CD4 truncated at the transmembrane exon (Fisher, R.A., et al., 1988, Nature 331:76-78).

6.2. RESULTS

6.2.1. USING INITIAL ASSAY PROTOCOL

Table IV shows the initial results of screening serum samples for released CD4 using OKT4 or OKT4A as capture reagent, and Leu3a as a detection reagent.

TABLE IV

CD4 DETECTION*

Sample	Capture Antibody: OKT4	OKT4A
HPB T Cell Lysate**		
5×10^6 cells/ml	0.895	0.139
2.5×10^6 cells/ml	0.769	0.079
1.25×10^6 cells/ml	0.549	0.046
T Cell Leukemia Patient Serum***		
Sample 1184	0.000	0.000
Sample 1174	0.000	0.020
Sample 1195	0.004	0.040
Sample 1147	0.004	0.000

* Values shown are OD_{490}, using the indicated capture antibody and biotinylated Leu3a as detection antibody.

** The indicated numbers of HPB (human leukemia T cell line) cells were lysed in 1 ml detergent buffer.

*** Serum from patients with acute HTLV I associated T cell leukemia

While this assay could detect solubilized CD4 in cell lysates, no detectable soluble CD4 was observed in the sera of patients with HTLV I associated T cell leukemia, which is a disease characterized by an intense activated population of T cells.

Subsequent efforts were focused on determining whether antibodies could be selected which might preferentially recognize a released (soluble) form of the CD4. Recombinant

CD4, with the transmembrane and cytoplasmic regions deleted at the gene level, was used as a model antigen. Antibodies were coated onto microtiter wells overnight and blocked as described. Samples containing either buffer, detergent solubilized CD4 from the Jurkat T cell line at two different dilutions, or recombinant CD4 were added, followed by a second incubation with HRP-conjugated antibodies (or biotinylated Leu3a followed by streptavidin HRP). Each antibody was evaluated on both a capture and detection mode with all other antibodies on each of the samples. The results are shown in Tables V and VI.

TABLE V

CD4 DETECTION IN CELL LYSATES*

	Capture Antibody				
Detection Antibody	3G2	B66.1	R2B7	B67.2	OKT4
3G2	0.204	0.227	0.154	0.242	>2.00/1.9
B66.1	0.053	0.064	1.420	0.053	>2.00/1.28
R2B7	0.230	>2.0/0.93	0.217	1.843	>2.09/>2
B67.2	0.027	0.030	0.110	0.008	1.50
OKT4	0.037	0.024	0.040	0.000	0.008
Leu3A	0.196	0.206	>2.00/1.94	0.279	>2.00/>2

* Cell lysates contained 5×10^6 cells/ml. Values shown are OD_{490}. For those antibody pairs where absorbance was >2.0 for 5×10^6 cells/ml lysate, the value for 1×10^6 cells/ml is shown in same box preceded by a slash.

TABLE VI

RECOMBINANT SOLUBLE CD4 DETECTION†

Detection Antibody*	Capture Antibody* 3G2	B66.1	R2B7	B67.2	OKT4
3G2	0.002	0.003	0.074	0.000	0.230
B66.1	0.002	0.012	0.170	0.004	0.010
R2B7	0.000	0.036	0.059	0.013	0.300
B67.2	0.000	0.000	0.008	0.000	0.009
OKT4	0.001	0.000	0.010	0.002	0.000
Leu3A	0.008	0.000	1.765	0.000	0.231

† Values shown are OD_{490}.
* mAb B53.1 was also used, but showed no positive results when used as either capture or detection reagent.

The data presented in Tables V and VI reveals a wide range of assay efficacies for the detection of solubilized cell-surface CD4 antigen (in cell lysate) or recombinant soluble CD4 antigen. Optimal combinations for detection of recombinant or lysate CD4 antigen are shown in Table VII.

TABLE VII

OPTIMAL PAIRS OF ANTIBODIES FOR DETECTION OF DETERGENT SOLUBILIZED OR RECOMBINANT CD4

Capture Antibody	Detection Antibody
R2B7	Leu3A
R2B7	B66.1
B66.1	R2B7
B67.2	R2B7
OKT4	R2B7
OKT4	B66.1
OKT4	B67.2
OKT4	Leu3A
OKT4	3G2

Interestingly, only the combination of R2B7 as a capture antibody with Leu3A as a detection antibody gave signal with the recombinant CD4 substantially equivalent to that seen in lysate, suggesting this pair might preferentially recognize soluble CD4.

Antibodies were generated from a mouse immunized with whole T cells and screened for their ability to replace Leu3a in an assay. 500 hybridoma clones were screened and three clones meeting the above criteria were identified. One of these clones, termed 8F4, showed the ability to block binding of FITC labeled Leu3A to CD4 positive T cell surfaces.

Antibodies 8F4 and R2B7 were evaluated with regard to optimal configuration in the assay. Table VIII shows that 8F4 used as capture antibody with R2B7 used as detection antibody produced a significantly greater ratio of signal

observed using recombinant soluble CD4 to signal observed using detergent solubilized membrane CD4, compared to the ratio of signal observed with R2B7 as a capture antibody and 8F4 as detection.

TABLE VIII

CD4 DETECTION

SAMPLE:	Absorbance at 490 nm: 8F4 Capture and R2B7 Detection	Absorbance at 490 nm: R2B7 Capture and 8F4 Detection
HPB Cell Lysate (cells/ml lysate)		
2×10^6	1.683	1.178
1×10^6	1.005	0.619
5×10^5	0.583	0.370
2.5×10^5	0.274	0.172
1.25×10^5	0.135	0.090
6.25×10^4	0.067	0.045
0	0.014	0.013
Recombinant Soluble CD4 Dilutions*		
1:2	>2.0	1.411
1:4	>2.0	0.991
1:8	>2.0	0.666
1:16	1.165	0.309
1:32	0.718	0.152
1:64	0.382	0.074
T Cell Culture Supernatants		
ST16** T cell line	0.187	0.135
5B4** T4 clone	0.152	0.104
6D11** T4 clone	0.171	0.120
5C8** T4 clone	0.331	0.246
TIL** 5C4 T8 clone	0.037	0.019

* Ratios represent the dilutions of cell culture supernatant of transfected CHO cells expressing the recombinant soluble CD4 antigen.

**Sample designation

The antibody used by Doumerc et al. (1986, 6th Intl. Congress of Immunology, Toronto, Ontario, Canada, July 6-11, 1986, Abstr. 5.54.6, p. 708), IOT4, was evaluated for its ability to measure the same form of CD4 antigen as that detected in the assay using 8F4 with R2B7. Table IX shows the results when IOT4 antibody was used as both capture and detection reagent as was done by Doumerc et al.

TABLE IX

CD4 DETECTION IN CELL LYSATE*

Capture Antibody		Detection Antibody 8F4	IOT4	R2B7
8F4	+NP40	.037	0.051	0.545
	-NP40	.013	0.035	0.423
IOT4	+NP40	.624	0.035	0.381
	-NP40	.063	0.017	0.040
R2B7	+NP40	ND	0.040	0.400
	-NP40	ND	0.049	0.320

RECOMBINANT SOLUBLE CD4 DETECTION*

Capture Antibody		Detection Antibody 8F4	IOT4	R2B7
8F4	+NP40	.003	.142	1.341
	-NP40	.005	.155	1.421
IOT4	+NP40	.004	.010	0.010
	-NP40	.006	.014	0.006
R2B7	+NP40	ND	.133	1.18
	-NP40	ND	.134	1.18

* Values shown are OD_{490}. Where indicated, 0.25% NP40 was present in both the sample diluent and conjugate diluent of the sandwich immunoassay. Recombinant CD4 assays were carried out using a standard dilution of cell culture supernatant of transfected CHO cells. ND: Not determined

Antibodies were evaluated for CD4 detection using different combinations of capture and detection reagents in assay matrices. Assays were carried out using 25% fetal calf serum in tris buffered saline, with and without Nonidet P40 (NP40) in both sample and conjugate diluents (Table IX). IOT4 reacted with detergent solubilized CD4 but failed to react with the recombinant soluble CD4. Also shown in Table IX are antibodies 8F4 and R2B7 in combination with themselves and IOT4. When used as a capture reagent, IOT4 detected only CD4 in cell lysates. Interestingly, however, when IOT4 was used as a detection reagent with 8F4 or R2B7 used as capture reagent, a much stronger signal is seen for the recombinant CD4 antigen than is seen with solubilized CD4 in cell lysates. It should be noted that this signal (for recombinant CD4) is significantly less than the signal obtained when R2B7 is paired with 8F4 as either detection or capture reagent. R2B7 when paired with itself was capable of a strong signal for both recombinant and cell lysate samples. Inclusion of NP40 failed to disrupt this signal. In contrast, 8F4 did not show such behavior, reacting only weakly with both cell lysate and recombinant material when used in both parts of the sandwich. IOT4 also failed to give a significant signal when paired with B66.1 and B67.2 for both cell lysate and recombinant samples.

Table X shows the results of screening culture supernatants from T cell lines or clones derived from patients with rheumatoid arthritis or lung cancer.

TABLE X

SOLUBLE CD4 AND CD8 DETECTION

Sample Designation	Phenotype	Soluble CD8 (U/ml)*	Soluble CD4 (U/ml)*
1	$CD4^+$ clone	112	249
2	$CD4^+$ clone	102	241
3	$CD4^+$ clone	111	277
4	$CD8^+$ clone	2,388	
5	$CD4^+$ clone	4,000	90
6	$CD4^+$, $CD8^+$ clone	63	70
TI1 5	$CD4^+$, $CD8^+$ mixed line	568	61
TI1 6	$CD4^+$, $CD8^+$ mixed line	4,000	238
TI1 6 2	$CD4^+$, $CD8^+$ mixed line	4,000	120
TI1 7 PBT	$CD4^+$, $CD8^+$ mixed line		51
TI1 4	B cell line		
5B4	$CD4^+$ clone		120
6G7	$CD4^+$ clone		240
5A1	$CD4^+$ clone		223
5C8	$CD4^+$ clone		115
6G7	$CD4^+$ clone		193
6D11	$CD4^+$ clone		116
5B4	$CD4^+$ clone		150
6D2	$CD4^+$ clone	53	176
5C4	$CD4^+$ clone		117
ST1 2H1	$CD4^+$ clone	52	178
ST1 1C10	$CD4^+$ clone		160
ST1 13G11	$CD8^+$ clone	3,217	
ST2 13C6	$CD4^+$ clone		132
ST2 13C6 2	$CD4^+$ clone		53
ST2 11C12	$CD8^+$ clone	1,046	
ST2 13A10	$CD4^+$ clone	369	94

ST2 CM	$CD4^+$, $CD8^+$ mixed line	221		
ST2 13H1	$CD8^+$ clone	3,428		
ST2 14A5	$CD4^+$ clone	72	142	
ST2 13A5	$CD4^+$ clone	71	161	
ST 13 PB	$CD4^+$, $CD8^+$ mixed line	4,000	50	
ST 16	$CD4^+$, $CD8^+$ mixed line	901	247	
ST5 PBT	$CD4^+$, $CD8^+$ mixed line	240		
ST5	$CD4^+$, $CD8^+$ mixed line			
TI14 PBT	$CD4^+$, $CD8^+$ mixed line	4,000	71	
TI14 10F8	$CD4^+$, $CD8^+$ mixed line	1,217	70	
ST12	$CD4^+$, $CD8^+$ mixed line	4,000		
ST11	$CD4^+$, $CD8^+$ mixed line	4,000	139	

* Blank values indicate undetectable levels. CD4 units were defined in terms of the amount of absorbance of CD4 antigen found in a lysate of 10^3 Jurkat T cells in 1% NP40 buffer, as measured using 8F4 as capture and R2B7 as detection reagents. CD8 units were based on a reference preparation of culture supernatant from Jurkat T cells used to standardize the CELLFREE® T8 (T Cell Sciences, Cambridge, MA) assay.

Cell phenotype was determined by flow cytometry. Soluble CD4 was determined using 100 µl cell culture supernatant in a single-step assay using R2B7 as the antibody immobilized on the solid phase with biotinyl Leu3a and streptavidin peroxidase used for detection. A commercially available sandwich immunoassay kit (CELLFREE® T8 Test Kit, T Cell Sciences, Inc., Cambridge, MA) was used to measure soluble CD8. The CD8 antigen detected by this assay has been characterized previously as a 52-55 kD dimer composed of monomer polypeptides, each with a molecular weight of approximately 27 kD. As shown in Table X, 21 of 21 $CD4^+$ clones showed soluble CD4 in the supernatant. Zero of four $CD8^+$ clones showed soluble CD4 in the supernatant. The cell

lines showed varying mixes of soluble CD4 and soluble CD8. No correlation was observed between cell number and level of soluble CD4.

Table XI shows the rate of release of CD4 into the media after in vitro stimulation of peripheral blood mononuclear cells.

TABLE XI

SOLUBLE CD4 LEVELS AFTER IN VITRO CELL STIMULATION

Experiment #	Type of Cell Stimulation*	Days in Culture	Soluble CD4 (U/ml)**
1	PHA	1	4.4
		2	6.4
		3	15.0
		4	17.4
1	OKT3	1	1.8
		2	4.4
		3	7.6
		4	11.5
1	NONE	1	1.1
		2	1.1
		3	4.8
		4	<1.0
2	PHA	1	7.6
		3	24.3
		4	43.9
		5	41.6

2	Phorbol myristate, acetate plus ionophore A2317	1	10.3
		3	12.9
		4	12.7
		5	7.8

* Carried out as described in Section 23.1.3, supra.

** CD4 units were as defined for Table XXIV.

In experiment #1, PHA showed significantly greater effect than stimulation with OKT3. Similarly, in experiment #2, PHA was significantly more effective than phorbol esters and ionophores at inducing CD4 release. No significant release occurred when cells were put into culture without mitogen, suggesting release is an active process and not merely due to cell death.

Table XII shows levels of soluble CD4 detected in sera of individuals with HTLV-1 associated adult T cell leukemia.

TABLE XII

DETECTION OF SOLUBLE CD4 IN PATIENT SERA

Sample Designation	Disease	Soluble CD4 (units/ml)*
1165	Acute Adult T Cell leukemia	3.51
1166	Acute Adult T Cell leukemia	37.56
1167	Acute Adult T Cell leukemia	7.90
1168	Acute Adult T Cell leukemia	5.61
1169	Acute Adult T Cell leukemia	1.41
1170	Acute Adult T Cell leukemia	1.07
1171	Acute Adult T Cell leukemia	0.08
1172	Acute Adult T Cell leukemia	0.52
1173	Acute Adult T Cell leukemia	9.41
1174	Acute Adult T Cell leukemia	9.54
1175	Chronic Adult T Cell leukemia	8.92
1176	Chromic Adult T Cell leukemia	0.66
1177	Chronic Adult T Cell leukemia	2.54
1178	Chronic Adult T Cell leukemia	0.23
1179	Chronic Adult T Cell leukemia	0.31
1180	Chronic Adult T Cell leukemia	0.23
1181	Chronic Adult T Cell leukemia	1.83
1182	Chronic Adult T Cell leukemia	3.36
1183	Chronic Adult T Cell leukemia	2.21
1184	Chronic Adult T Cell leukemia	3.78
1185	Smoldering Adult T Cell leukemia	0.87
1186	Smoldering Adult T Cell leukemia	0.59
1187	Smoldering Adult T Cell leukemia	0.24
1188	Smoldering Adult T Cell leukemia	0.31
1189	Smoldering Adult T Cell leukemia	1.34
1190	Smoldering Adult T Cell leukemia	1.01
1191	Smoldering Adult T Cell leukemia	1.61
1192	Smoldering Adult T Cell leukemia	0.91

* Detected using R2B7 as capture reagent and biotinylated Leu3A as detection reagent. CD4 units were as defined for Table X.

Individuals with the most active stages of the disease had the highest levels of soluble CD4 in their sera.

Figure 1 shows the levels of CD4 in sera of normal individuals and in patients from a number of disease groups. Levels of CD4 in synovial fluid of rheumatoid arthritis patients and in sera of lung cancer patients were elevated as compared to the levels in sera from normal individuals.

Table XIII shows CD4 levels in longitudinal samples from patients on IL-2 therapy.

TABLE XIII

SOLUBLE CD4 LEVELS IN PATIENTS UNDERGOING IL-2 THERAPY*

Patient Designation	Date (mo./day)	Soluble CD8 (U/ml)	Soluble CD4 (U/ml)	Soluble IL2R (U/ml)
A	4/27	184	7	1302
A	5/04	492	19	>1600
A	5/06	531	28	>1600
A	5/10	529	27	>1600
A	5/11	490	22	>1600
B	1/29	325	10	385
B	2/04	595	19	>1600
B	2/10	1221	12	>1600
B	2/12	914	17	>1600
B	2/20	452	16	>1600
C	1/05	319	21	964
C	1/15	1232	26	>1600
C	1/21	890	26	>1600
D	1/15	269	10	294
D	3/04	271	7	>1600
D	3/09	519	16	>1600
D	3/10	484	10	>1600
E	3/23	621	14	>1600
F	3/22	315	165	627
F	3/29	615	102	>1600
F	4/05	898	140	>1600
Patient 1		333	9	494
Patient 2		222	22	>1600
Patient 3		274	12	683
Patient 4		149	11	650
Patient 5		1492	49	>1600
Patient 6		1008	74	>1600
Patient 7		608	8	>1600
Patient 8		2094	27	>1600
Patient 9		779	16	>1600
Patient 10		1400	31	>1600
Normal 1**		476	9	466
Normal 2**		311	6	540
Normal 3**		178	3	396
Normal 4**			5	
Normal 5**			3	
Normal 6**			4	
Normal 7**			6	
Normal 8**			6	
Normal 9**			8	
Normal 10**			3	

* Soluble CD8 was measured using the CELLFREE T8 Test Kit (T Cell Sciences, Cambridge, MA). Soluble CD4 was detected using 8F4 capture, R2B7 detection. CD4 units were as defined for Table XXIV. Soluble IL2R was measured using the CELLFREE IL2R Test Kit (T Cell Sciences, Cambridge, MA).

**Healthy blood donors (not undergoing IL-2 therapy)

The data of Table XIII shows that detectable levels of soluble CD4 are present in sera of patients being treated with IL-2. One of the events observed in IL-2 therapy is an increase in circulating activated CD4 positive lymphocytes. Soluble CD4 levels in these patients fluctuate throughout the course of therapy and may have prognostic value.

Table XIV shows levels of soluble CD4, along with soluble IL2R, in renal transplantation patients.

TABLE XIV

SOLUBLE CD4 LEVELS IN RENAL TRANSPLANTATION PATIENTS

Patient Designation	Sample Date (mo./day)	Soluble CD4* (U/ml)	Soluble IL2R† (U/ml)
P	4/07	6.4	455
	4/09	7.6	493
	4/14	20.4	4090
	4/16	9.3	3865
	5/02	6.8	1200
	5/30	37.4	565
H	3/14	16.4	811
	3/21	11.7	441
	4/09	12.4	347
	5/14	21.6	692
	6/11	36.9	907
L	6/15	91.8	1965
	6/16	29.2	2705
	6/23	38.1	3990
	6/30	48.8	7400
	7/07	35.2	6300
G	4/30	26.1	1288
	5/05	7.6	700
	5/07	24.2	1845
	5/09	32.6	3625
	5/12	17.1	3635
	5/14	18.8	3035
	5/16	21.2	3040
	5/21	20.0	4080
	6/04	46.5	2475
	6/25	19.4	1995
S	6/13	6.6	1090
	6/16	13.7	680
	6/18	13.1	930
	6/20	11.1	1705
	6/23	8.3	2708
	6/25	19.5	5515
	6/27	11.2	3460
	6/30	9.9	2205
	7/07	12.8	1825

* Detected using 8F4 capture, R2B7 detection
† Analyzed using CELLFREE® IL2R Test Kit

Elevated levels of CD4 did not show a correlation with IL2R but did, like IL2R, show increases during rejection episodes.

6.2.2. RESULTS USING OPTIMIZED ASSAY PROTOCOL

Once the soluble CD4 assay was optimized and any effects of rheumatoid factor eliminated, it was possible to detect much lower levels of soluble CD4. For normal healthy individuals, the range of soluble CD4 was 8 U/ml to 36 U/ml with a mean of 17.2 U/ml. This was determined from the assay of 189 normal samples. A high number of replicates was also run to achieve confidence at the low end of the range.

Using this improved assay, a series of renal transplant patients was analyzed, and the data is presented in Table XV.

TABLE XV

RENAL TRANSPLANT PATIENTS

PATIENT	Diagnosis[1]	Soluble$_2$CD4 (U/ml)[2]	Soluble$_3$IL-2R (U/ml)[3]	Soluble$_4$CD8 (U/ml)[4]
C1	CsA	69	200	181
	CsA	30	340	448
B1	Rejection	15	240	47
Z1	Stable	19	380	310
	Stable	19	390	374
	Stable	15	300	321
R1	Rejection	24	420	481
	Rejection	33	820	120
M1	CsA	122	770	534
	CsA	29	330	570

	CsA	26	80	526
L1	Rejection	43	1040	ND
	Rejection	30	1120	ND
S1	Rejection	25	680	ND
P1	Rejection	44	2180	1894
	Rejection	25	370	390
S2	CsA	117	510	267
	CsA	22	390	67
A1	Stable	96	400	731
	Stable	27	320	390
	Stable	23	40	409
L2	Rejection	23	1240	228
	Rejection	too high	1000	1559
	Rejection	24	1050	452

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[1] Diagnosis was either cyclosporin A toxicity (CsA), stable transplant, or rejection; multiple patient serum samples were taken at different times.

[2] Soluble CD4 was measured using the improved assay (Section 6.1.2.2, supra).

[3] Soluble IL2R was measured using the CELLFREE® IL-2R Test kit (T Cell Sciences, Cambridge, MA).

[4] Soluble T8 was measured using the CELLFREE® T8 Test kit (T Cell Sciences, Cambridge, MA).

ND: Not Determined.

The above data indicates that it was possible to detect soluble CD4 levels in renal transplant patients in the phases of rejection, toxicity and stability. It was also possible to detect elevated levels of other soluble T cell surface markers, such as soluble CD8 and soluble IL2R. This data shows that soluble receptors are present during the course of transplant episodes. It is expected that a longitudinal study of each of these patients will provide data that will indicate how the levels of each soluble marker change with toxicity, rejection or stability episodes. Thus, a change in the observed level for any particular marker, such as an increase or decrease or no change, may be of more value than the absolute level of a

marker present at any one point in time, for the diagnosis or monitoring of treatment in disease (see Section 8, infra). For comparison, the change in the observed level of a marker must be compared to a baseline level which could either be the level seen in normal individuals with no disease, the pre-transplant level in the renal patient, the value present in a stable situation or during remission of symptoms, etc.

In a preferred embodiment, the diagnosis of disease or monitoring of treatments of patients with renal transplants or other diseases and states of immune activation will be through an analysis of a panel of soluble T cell markers, rather than from only one individual marker. Thus, for example, a better prognostic indicator can be a rise in one marker relative to a simultaneous fall in another marker. The resulting profile of soluble T cell marker expression should be an exquisite indicator of minute changes in the immune system as its function is modified by therapeutic treatments or disease progression (see Section 7, infra).

Table XVI gives the values of soluble CD4 levels seen during preliminary studies on patients with Acquired Immune Deficiency Syndrome (AIDS) and other stages of HIV-induced disease including Kaposi's Sarcoma (KS), AIDS related complex (ARC) or asymptomatic seropositive (ASYM).

TABLE XVI

LEVELS OF SOLUBLE RECEPTORS IN HIV-INFECTED PATIENTS

PATIENT	Diagnosis	Soluble CD4* (U/ml)	Soluble IL-2R* (U/ml)	Soluble CD8* (U/ml)
1	AIDS	10	699	1236
2	AIDS	10	792	2351
3	AIDS	20	1092	1099
4	AIDS	21	506	1706
5	AIDS	24	2105	1104
6	AIDS	56	1340	753
7	AIDS	25	2099	1440
8	AIDS	1	1760	1007
9	AIDS	20	1964	626
10	AIDS	3	1079	306
11	AIDS	1	1529	343
12	AIDS	13	1025	647
13	AIDS	-1	2014	438
14	AIDS	16	747	507
15	ARC	71	1645	2646
16	ARC	23	967	7331
17	ASYM	11	489	487
18	ARC	23	563	4073
19	ARC	177	1508	1246
20	ARC	-0	978	572
21	ARC	15	2093	1731
22	ARC	16	1742	1279
23	ASYM	15	740	2700
24	ASYM	28	1403	1583
25	ASYM	24	1392	3037
26	ASYM	13	1084	2290
27	ASYM	12	558	1915
28	ASYM	19	1182	5580
29	ASYM	14	1308	1702
30	ASYM	16	806	430
31	ASYM	17	1012	1386
32	ASYM	13	1541	5127
33	ASYM	42	1752	4022
34	ASYM	10	504	1598

* Assays as indicated for Table XV.

From the above data, it is clear that although the values of soluble CD4 are low compared to the other soluble marker levels, they are easily detectable in sera from patients having different stages of HIV-induced disease. As discussed supra, improved prognostic indices based upon levels or changes in levels of these soluble markers are expected with longitudinal studies involving a panel of soluble receptor markers. Such studies should reveal a profile of soluble receptors that can be used to determine the stage of progression towards AIDS in patients or the response to treatment of such patients. A longitudinal study of the patients in Table XVI and others can be conducted to determine the soluble receptor profiles of the patients and to correlate these profiles with the efficacy of their ongoing azido-deoxythymidine treatment (see Section 8, infra).

Using the improved assay format, paired samples of synovial fluid and serum were analyzed for several patients with rheumatoid arthritis. This data is presented in Table XVII.

TABLE XVII

DETECTION OF SOLUBLE CD4 IN PAIRED SYNOVIAL FLUID AND SERUM SAMPLES FROM INDIVIDUAL PATIENTS

PATIENT	Sample[1]	Soluble $CD4_2$ (U/ml) + HA[2]	Soluble $CD4_3$ (U/ml) + IgG[3]
1	serum	16	13
	fluid	42	36
2	serum	15	12
	fluid	64	57
3	serum	15	14
	fluid	44	39

4	serum	21	16
	fluid	103	96
5	serum	23	19
	fluid	66	59
6	serum	19	14
	fluid	41	37
7	serum	22	22
	fluid	78	84
8	serum	15	13
	fluid	80	73
9	serum	15	13
	fluid	87	90
10	serum	19	15
	fluid	129	125

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1 Samples were obtained from the serum or synovial fluid of each patient.

2 HA = heat aggregated IgG added to remove any false positive problems associated with occassional high RF (rheumatoid factor) containing samples.

3 IgG = unaggregated IgG control to detect samples that may have had RF problems.

The above data indicates that with the increased sensitivity of the employed assay, it was possible to detect soluble CD4 levels in both serum and synovial fluid samples of rheumatoid arthritis patients. The levels observed in serum samples were within the normal range, however. Furthermore, the levels of soluble CD4 were elevated in synovial fluid samples relative to serum samples form 10 of 10 patients analyzed. This suggests a localized production and release of CD4 antigen.

Levels of soluble CD4 in sera of normal individuals and patients from a number of disease groups were measured, with the results shown in Figure 2. Elevated levels of soluble CD4 were observed in renal transplant patients, synovial fluid of rheumatoid arthritis patients, in some patients with EBV infections, and in patients with various leukemias.

6.3. DISCUSSION

Assays have been described herein that allow the measurement of CD4 in a detergent solubilized membrane form, in a recombinant soluble form (genetically engineered to exclude the transmembrane region), and in a spontaneously released form from activated T cells. Nine different anti-CD4 antibodies, in a total of 63 different configurations (one configuration consisting of a single capture and a single detection antibody) were screened for suitability for detecting CD4 in cell lysates. Nine such suitable configurations were identified. Of these, only five configurations showed significant reactivity with soluble recombinant CD4. Three of these five configurations involved R2B7 as one of the antibodies. R2B7 when paired with 8F4 showed greatest sensitivity for detecting soluble CD4 both from recombinant and natural sources. The greatest ratio of signal from a soluble recombinant CD4 to signal from solubilized lysate CD4 is seen using 8F4 capture with R2B7 detection. This is roughly twice the ratio seen in the reverse configuration. It is possible that binding of one or more of the antibodies induces conformational changes in the molecule. This is supported by the observation that when IOT4 is used as a capture reagent, only solubilized lysate CD4 may be detected (using 8F4 or R2B7 detection), whereas when 8F4 or R2B7 are used as capture in combination with IOT4 as detection, soluble CD4 is preferentially detected over solubilized lysate CD4. Recombinant CD4 serves as a good model for released CD4 since those antibody combinations which work best with recombinant soluble CD4 also work best with the spontaneously soluble form of the molecule. Thus, selection of the antibodies and their configuration in the sandwich immunoassay is crucial to successful detection of released CD4.

Doumerc et al. (1986, 6th Intl. Congress of Immunology, Toronto, Ontario, Canada, July 6-11, 1986, Abstr. 5.54.6, p. 708) have described an enzyme immunoassay based on the use of IOT4 as a capture and detection reagent to detect CD4 in serum. In our hands, this configuration works only to measure the membrane form of the molecule and fails to adequately measure the soluble recombinant or soluble spontaneously released form of the molecule described here. Doumerc et al. (id.) disclose increased serum CD4 during transient episodes of lymphocyte destruction. It is possible that the form of the molecule measured by Doumerc et al. represents a form still associated with pieces of membrane, not a truly soluble moiety. Such a membrane form will tend to aggregate or associate into micelles or vesicles, thus rendering it capable of detection in a sandwich immunoassay using the same antibody as both capture and detection reagent. Since soluble CD4 is not known to exist in multimeric structures, in the absence of repeating epitopes, the same antibody cannot be used as both capture and detection reagent for assay of soluble CD4. Doumerc et al. further suggest that the CD4 they measure correlates with total CD4 cell pool. We suggest that CD4 release, like CD8, is rather a function of activation of cells. This is supported by the kinetics of released CD4 observed during in vitro stimulation. Thus, the molecule described by Doumerc is significantly different from the molecule described herein.

CD4 release may be a function of the type and pathway of activation. Phytohemagglutinin and T3 stimulation both resulted in a release of small amounts of CD4. Stimulation with phorbol esters, known to cause phosphorylation and internalization of CD4, or with ionophores, resulted in significantly less released CD4 than did PHA stimulation, despite intense cellular activation. The kinetics of CD4 release were also significantly different between cells

stimulated by phorbol esters and those stimulated by PHA. Release can also not be attributed to simple membrane turnover. No CD4 is released by resting cells in vitro.

Cloned IL-2 dependent $CD4^+$ T cells or T cell lines containing $CD4^+$ cells all showed detectable soluble CD4 in their culture supernatants. $CD8^+$ cells showed only soluble CD8. Thus, the released molecules are an accurate reflection of the cell surface phenotype of the cells. No correlation was observed between levels of soluble CD4 and the number of cells.

Low levels of CD4 were seen in sera from normal individuals. Elevated soluble CD4 was observed in certain individuals with EBV infection, lung cancer and with T cell leukemias, and correlated overall with the stage of disease (Fig. 1,2). Elevations in CD4 antigen levels were also observed in disorders due to HIV infection, and in some synovial fluid, but not sera, from patients with rheumatoid arthritis. Elevations were observed in certain patients on IL-2 therapy where there is activation of $CD4^+$ as well as $CD8^+$ cells and natural killer cells. Finally, elevations were observed in sera of some patients undergoing renal allograft rejection or cyclosporin A toxicity. Soluble CD4 levels may thus be of value in the diagnosis and monitoring of a pathologic event.

The relationship between spontaneously released CD4 and membrane CD4 can be determined from patterns of antibody reactivity. If the spontaneously released material were identical to the cell-surface polypeptide, it should behave in the assays, which have detergent incorporated into them, like solubilized CD4 in cell lysate. If they are more analogous to the recombinant truncated version of CD4 they should behave like it. The latter is the case; that is, those antibody pairs which afford suitable detection of solubilized lysate CD4, but not recombinant soluble CD4, yielded poor detection of the soluble CD4 from T cell

culture supernatant, whereas those antibody pairs showing optimal reactivity with recombinant soluble CD4 also reacted optimally with the released material. It is clear from antibody reactivity patterns that the released form of the CD4 antigen differs significantly from the membrane form.

A key element in deriving a successful sandwich immunoassay for the detection of soluble CD4 was the strategy employed in antibody selection. A readily available model source of soluble CD4, recombinant truncated CD4, was used in a screening procedure to select antibodies with a preferential ability to identify the released form of the molecule. Using this criteria, a pair of antibodies was selected that could detect spontaneously released CD4 in sera and in culture supernatants. This strategy may be widely applicable to the detection and discovery of other released molecules.

7. EVALUATION OF THE ROLE OF THE SOLUBLE CD8 RECEPTOR AND IL-2 RECEPTOR IN PATIENTS WITH HUMAN IMMUNODEFICIENCY VIRUS INFECTION

We evaluated the levels of soluble CD8 and IL2R in patients with HIV infection. The levels of CD8 and IL2R were compared with each other and with levels of plasma p24 antigen, $CD4^+$ cells, $CD8^+$ cells, and CD4/CD8 ratios.

7.1. METHODS

7.1.1. SAMPLE SELECTION

Stored serum from patients with HIV infection was examined using either the CELLFREE® IL-2R Test Kit or CELLFREE® T8 Test Kit (T Cell Sciences, Cambridge, MA). 63 patients with HIV infection and 7 normal controls were studied. Patients were divided into 4 groups depending upon the manifestations of HIV infection; these groups were: 21 assymptomatic seropositive (ASSYM), 19 AIDS related complex (ARC), 13 Kaposi's sarcoma (KS) and 10 AIDS with

opportunistic infection. Stored sera from 15 patients followed longitudinally over a period of 2 years were also examined.

7.1.2. HIV p24 ANTIGEN ASSAY

This assay utilized a sandwich ELISA microplate format. Highly specific rabbit polyclonal antibodies to HIV p24 core antigen were immobilized on microtiter plate wells and used to capture HIV p24 core antigen present in 450 µl of plasma. The captured HIV p24 core antigen was complexed with biotinylated polyclonal antibodies to HIV p24 core antigen and probed with a streptavidin-horseradish peroxidase conjugate. This complex was visualized by incubation with orthophenyldiamine-HCl producing a color intensity directly proportional to the amount of HIV p24 core antigen captured. The results were also quantitated spectrophotometrically and compared against the absorbance of an HIV p24 core antigen standard curve.

7.1.3. CD4/CD8 RATIO

The CD4 and CD8 ratios were determined by standard flow cytometry.

7.2. RESULTS

Soluble CD8, soluble IL2R, p24 antigen, CD4/CD8 ratio, $CD4^+$ cells and $CD8^+$ cells were measured in samples from patients with AIDS, ARC, KS, ASSYM or normals, as shown in Table XVIII.

TABLE XVIII

Measurement of Shed Receptors in Patients with Different Manifestations of AIDS*

Patient Group	Soluble CD8	Soluble IL2R	p24	CD4/CD8	%CD4	%CD8
ASSYM	1029 ± 89	783 ± 98	16.4 ± 9.0	0.73 ± 0.08	27.9 ± 2.9	50.2 ± 3.1
KS	1165 ± 131	970 ± 129	8.9 ± 2.3	0.59 ± 0.12	14.7 ± 1.6	50.0 ± 6.4
ARC	980 ± 118	1011 ± 119	9.7 ± 3.5	0.71 ± 0.11	28.3 ± 3.6	47.6 ± 2.9
AIDS	717 ± 115	1566 ± 157	19.7 ± 8.1	0.19 ± 0.05	9.9 ± 2.3	50.2 ± 3.4
Normals	508 ± 40	188 ± 28				

* Expressed as mean + standard mean;
soluble CD8 and soluble IL2R expressed as units/ml;
p24 expressed as pg/ml

Using chi-square analysis, comparing each group with normal controls, the majority of patients with AIDS, ARC, ASSYM and KS showed levels of both IL2R and soluble CD8 which were greater than the upper 95% value of normal ($p<0.00001$). IL2R was better than CD4/CD8, %CD4, and p24 for discriminating ASSYM from AIDS ($p<0.0001$), ASSYM from ARC ($p<0.002$) and ARC from AIDS ($p<0.0001$). Of interest is the observed difference in soluble CD8 between groups with ASSYM, KS and AIDS. This suggests that early on in the course of HIV infection, elevated soluble CD8 levels may reflect host immune response to HIV. It has been demonstrated that CD8 positive cells are able to control HIV infection in vitro by suppressing viral replication (Walker, C.M. et al., 1986, Science, 234, 1563-1566). Soluble CD8 levels may be an accurate measure of the immune system's attempts to suppress HIV infection. In addition, by using a combination of both soluble IL2R and soluble CD8 levels in each patient group, it was possible to distinguish between normal and assymptomatic patients.

In addition to determining the value of each soluble marker level, a comparison was also made between different soluble marker values to determine how they correlated with one another. The data presented in Table XIX presents the correlation observed between several marker combinations. The samples analyzed for this table do not represent longitudinal samples obtained from individual patients, but samples from the population of people belonging to the different HIV-infected groups.

TABLE XIX

CORRELATIONS BETWEEN THE BEHAVIOR OF DIFFERENT PARAMETERS IN AIDS PATIENTS*

Correlation	ASSYM	ARC	KS	AIDS	Combined HIV Positive
sCD8 v sIL-2R		+			+
sCD8 v CD4/CD8	-	-		+	-
sCD8 v % CD4		-			
sCD8 v % CD8	+				+
p24 v sIL2R	+				+
sIL2R v CD4/CD8		-			-
sIL2R v % CD4		-			-
sIL2R v % CD8		+			
CD4/CD8 v % CD4	+	+		+	+
CD4/CD4 v % CD8	-	-			-
% CD4 v % CD8	-	-			-

* Pearson Correlation is expressed as + (positive correlation between the two parameters) or - (negative correlation between the two parameters); blank values indicate that the correlation had a probability index > 0.05; s = soluble

It is clear that the occurrence of soluble markers is not independent of one another or of other markers of the immune system. Thus, the combined behavior of these receptors should be even more valuable than the observance of any single receptor.

In addition to the patient samples analyzed above, three patients with ARC and three with KS were followed longitudinally as shown in Figure 3. The results of this study indicated that soluble CD8 levels appeared to parallel

th change in p24 core antigen levels. Since the p24 core antigen levels have not proved to be sensitive enough tests of the progression of AIDS, we propose that the soluble CD8 levels, which reflect the status of the immune system itself, can be a much better indicator.

8. A CRITICAL ANALYSIS OF THE DIAGNOSTIC UTILITIES OF IMMUNOASSAYS FOR SERUM AND URINE SOLUBLE INTERLEUKIN-2 RECEPTOR LEVELS IN RENAL ALLOGRAFT RECIPIENTS

As described herein, a study was conducted to evaluate the diagnostic utility of assays for serum and urine soluble IL2R in renal allograft recipients. Serial serum and urine samples obtained prospectively were tested for soluble IL2R levels by sandwich enzyme immunoassay test, and correlations were sought with serum creatinine and episodes of rejection, cyclosporin A (CsA) toxicity, and infections. Our results demonstrated that a rise in serum IL2R between samples taken within a week predicted the onset of rejection better than absolute serum IL2R levels or urine values. For the diagnosis of acute rejection, a rise in serum IL2R (sensitivity 73%, specificity 87%) was comparable in overall test performance with a rise in serum creatinine (sensitivity 70%, specificity 84%). Overall, the two tests had equivalent receiver operating characteristic curves. Because the etiology of false positives in creatinine and IL2R assays differed (primarily cyclosporine toxicity and infection, respectively), the predictive value of the combined tests was superior to either alone.

8.1. MATERIALS AND METHODS

8.1.1. PATIENTS

The study population consisted of 33 adults who received renal allografts at the Massachusetts General Hospital. Maintenance immunosuppression consisted of

cyclosporin A (CsA) and prednisone (Colvin, R.B., et al., 1987, Clin. Immunol. Immunopathol. 43:273-276). Episodes of rejection were treated with increased steroids, anti-T3 monoclonal antibody OKT3, or ATG (anti-thymocyte globulin) (Delmonico, F.L., et al., 1987, Am. Surg. 206:649-654). Rejection was diagnosed by a progressive rise in serum creatinine that responded to increased immunosuppression. Other causes of renal failure were excluded. Twenty-one episodes of rejection in 15 patients were monitored during the six month study period. Biopsies were obtained in ten patients and showed acute cellular rejection in all instances (Colvin, R.B., et al., 1987, Clin. Immunol. Immunopathol. 43:273-276). CsA toxicity was diagnosed by a rise in creatinine that responded to decreased CsA dose. Nine toxicity episodes occurred in eight patients. Three viral episodes occurred in three patients: a severe cytomegalovirus infection, lymphoproliferation associated with Epstein Barr virus, and a transient gastroenteritis accompanied by fever and lymphocytosis.

8.1.2. IL2R ASSAY

Serum (N=481) and urine (N=274) samples were obtained whenever possible prior to transplant and serially 1-2 times per week during hospitalization and at most clinic visits for the first 3-6 months after transplantation. Samples were coded and stored frozen until assay. Soluble immunoreactive IL2R levels were assayed by a sandwich enzyme immunoassay test kit according to the specifications of the manufacturer (CELLFREE®, T Cell Sciences, Inc.) (Colvin, R.B., et al., 1987, Clin. Immunol. Immunopathol. 43:273-276). Assays were performed in batches calibrated to units based on a standard supernatant from phytohemagglutinin activated lymphocytes.

8.1.3. DATA ANALYSIS

After decoding and classification of the clinical status, the results of the serum and urine IL2R assays and the serum creatinine assays were compared according to standard statistical techniques, using a spread sheet data base. Samples taken within the first four days after transplantation and during treatment for rejection and for two days afterward were excluded from analysis. Various measurements (absolute and change between serial samples) were compared for their clinical utility, as judged by sensitivity, specificity, predictive value, and Receiver Operating Characteristic (ROC) curves (Fink, D.J. and Galen, R.S., 1982, in Computer Aids to Clinical Decisions, Vol. 2, Williams, B.T., ed., CRC Press, Cleveland, OH, pp. 1-65).

8.2. RESULTS

The aggregate data for serum and urine IL2R levels are summarized in Table XXXI. The concentration of serum IL2R in 24 pretransplant patients was elevated compared with normal controls, but fell after transplantation in 20 of these patients. Pretreatment IL2R levels from patients who had no rejection episodes did not differ from those with subsequent rejection episodes. In 4 of the 5 patients with delayed onset of function (creatinine not falling below 4 by day 7), the serum IL2R levels remained elevated longer (transiently falling on days 1-2 in two patients). Patients with no episodes of rejection during the monitoring period had moderately elevated serum IL2R levels (980 ± 692, 175 samples, 18 patients; $P < .001$) compared with normals. Samples that were taken during stable periods from the patients that had an episode of rejection were similar and have been included in the "stable" category in Table XXXI.

Serum IL2R usually rose during episodes of rejection and fell after successful antirejection immunosuppression was instituted. Figure 4 illustrates a representative patient. Seven patients with rejection episodes were tested 1-2 days before the first rise in serum creatinine. Four patients had a rise in serum IL2R greater than the 90th percentile of stable patients. Overall, the mean serum IL2R was elevated 1-2 days prior to the rise in creatinine ($P < .03$). Fourteen episodes of rejection in 9 patients were sampled on the first day of creatinine elevation; 9 of 14 samples (64%) were elevated above the 90th percentile of values during periods of stability. The IL2R levels remained elevated after the creatinine rise and into the treatment period, finally declining during or after antirejection therapy to the "stable" levels noted above. If only biopsy-confirmed rejection episodes are analyzed, the values are not significantly different (Table XXXI). Serum IL2R also rose during episodes of infection (CMV, EBV, gastroenteritis) (Table XXXI). In contrast, serum IL2R levels were not significantly raised in 8 patients with cyclosporine A toxicity.

The urine IL2R concentration rose in the immediate post-transplant period and remained higher in periods of stability than in pretransplant levels (Table XXXI). During episodes of rejection, urine IL2R rose in a pattern that was not distinguishable from the serum values, except that somewhat greater sample-to-sample variation was noted. Urine IL2R also followed the pattern of serum IL2R during episodes of infection and cyclosporine A toxicity.

TABLE XXXI

IL2R LEVELS IN RENAL ALLOGRAFT RECIPIENTS*

Status	Serum N**	Serum IL2R	p	Urine N	Urine IL2R	p
Pre-Transplant	24 (24)	1541±852		8 (8)	264±235	
Days 1-4	53 (31)	1386±874		36 (24)	636±567	
Stable	258 (31)	1218±961		117 (24)	586±427	
Rejection						
Before creatinine rise (1-2 d)	8 (7)	2075 ±1065	<.03	5 (3)	949±698	ns
biopsy	4 (4)	2629±991		3 (3)	1241±762	
First day of creatinine rise	14 (9)	2705±1410	<.001	9 (7)	1188±590	<.01
biopsy	7 (6)	2977±1580		6 (5)	1436±576	
After creatinine rise	19 (10)	2843±1887	<.001	11 (6)	1511±909	<.01
biopsy	9 (6)	3148±2422		11 (6)	1454±933	
On treatment	97 (15)	2870±1965	<.001	66 (11)	1646±951	<.001
Infection	15 (3)	16098±13587	<.001	7 (2)	3654±1830	<.01
CsA toxicity	14 (8)	990±594	ns	7 (6)	512±244	ns

* IL2R values are in units/ml (mean ± standard deviation); N, number of samples; number of patients in parentheses; p, comparison with stable patients, one-tail d t-test; ns, not significant (>.05). The data for the rejection episodes that were confirmed on biopsy are listed separately.

** Number of samples

The absolute concentration of serum IL2R had a sensitivity of 46.3% and a specificity of 87.1% for the diagnosis of rejection, using the 90th percentile of stable patients as the threshold (Table XXXII). Even lower sensitivity occurred with a higher threshold (17% using the 95th percentile). Urine IL2R had comparable sensitivity and specificity (44.0% and 86.4%, respectively). A single serum creatinine level was no better, using the 90th percentile as a threshold for a positive test (sensitivity 39.0%, specificity 88.9%).

TABLE XXXII

COMPARISON OF ASSAYS FOR THE DIAGNOSIS OF RENAL ALLOGRAFT REJECTION*

Assay	N**	Sensitivity (%)	Specificity (%)
Serum			
IL2R	328	46.3	87.1
Creatinine	328	39.0	88.9
Δ IL2R	225	73.3	86.7
Δ Creatinine	225	70.3	84.0
Urine			
IL2R	156	44.0	86.4
Δ IL2R	111	52.4	87.8

* Positive defined as >90th percentile of values from stable patients. Samples taken during the first 4 days after transplantation were excluded from this analysis. Rejection samples were taken from 2 days before the first rise in creatinine until just before anti-rejection therapy was begun. N, number of samples. Δ, rise in serial values taken within 1 week.

** Number of samples

In clinical practice, the absolute level of serum creatinine is less useful for the diagnosis of rejection than a rise in the level in serial samples. Based on this analogy, we determined whether the change in IL2R concentration in serial samples taken within a week (delta IL2R) might be more sensitive than the absolute level. The individual data on serum and urine IL2R and serum creatinine are given in a dot plot (Fig. 5), and are summarized in Table XXXII. The rise in serum IL2R had a much greater sensitivity (73.3%) than absolute serum IL2R levels for the diagnosis of rejection with no loss of specificity (86.7%). The delta urine IL2R measurement was not appreciably better than the absolute urine level and less sensitive (52.4%) than the delta serum IL2R (Table XXXII).

A rise in serum IL2R was comparable in sensitivity and specificity to a rise in serum creatinine using the 90th percentile of stable patients to define a positive test (Table XXXII). The two tests also had a comparable sensitivity specificity relationship overall, independent of the threshold selected for definition of a positive test, as shown in the ROC curves (Fig. 6). However, because the sources of false positives in creatinine and IL2R assays differ (primarily cyclosporine A toxicity and infection, respectively), the predictive value of the combined tests was superior to either test alone (Table XXXIII).

TABLE XXXIII

PREDICTIVE VALUE OF SERIAL SERUM IL2R AND CREATININE ASSAYS

Serum Values			% of Samples with Each Diagnosis			
Δ Cr >0.1 mg/dl	Δ IL2R >139 U/ml	N**	Stable	Rejection	CsA Toxicity	Infection
Single Test:						
-		169	87.2	7.1	2.6	5.1
+		56	33.9	44.6	14.3	5.4
	-	173	85.3	6.1	6.7	1.8
	+	52	30.8	51.9	1.9	15.4
Combined Tests:						
-	-	143	92.3	3.5	2.8	1.4
+	+	26	7.7	80.8	3.8	7.7
+	-	30	56.7	16.7	23.3	3.3
-	+	26	53.8	23.1	0.0	23.1
Overall		225	73.3	16.4	5.3	4.9

* Rise in values 5 days to 6 months after transplantation between serial samples taken within 1 week. Rejection samples were taken from 2 days before the rise in creatinine until the institution of anti-rejection therapy. Positive values were defined as >90th percentile of stable values.

**N, Number of samples

8.3. DISCUSSION

The development of a reliable, noninvasive, economical diagnostic test for immunologic rejection is an important goal. The renal biopsy is the reference standard, but is not without risk and expense. Fine needle aspirates have a lower risk, but have not been widely applied because they require specialized expertise for performance and interpretation. Many less invasive blood tests have been proposed, only to be abandoned due to a high cost/benefit ratio or lack of clinical predictive value beyond that of serum creatinine.

We have found that the specificity and sensitivity of soluble serum IL2R assays are similar to those of serum creatinine for the diagnosis of rejection. Among the various IL2R measurements, the serial change in serum IL2R was the most sensitive and specific. The absolute level of serum IL2R varied more from patient to patient and was elevated in chronic renal failure (Colvin, R.B., et al., 1987, Clin. Immunol. Immunopathol. 43:273-276). The urinary concentration of IL2R showed a similar pattern, but had greater sample to sample variation, probably due in part to differences in urine output and urinary degradation of the immunoreactive determinants. Standardization with urine creatinine concentration and inhibition of protease activity can be used to improve the quality of the urinary assays.

A revealing analysis of a diagnostic test is its ROC curve, which formalizes the relationship between sensitivity and specificity, and is an intrinsic property of the test, independent of disease prevalence or the definition of a positive result (Fink, D.J. and Galen, R.S., 1982, in Computer Aids to Clinical Decisions, Vol. 2, Williams, B.T., ed., CRC Press, Cleveland, OH, pp. 1-65). ROC curves reported in these studies demonstrate that the overall test performance of serum IL2R was indistinguishable from serum

creatinine for the diagnosis of rejection. However, each test had different sources of "false positives" in the allograft recipients. CsA toxicity raised the creatinine levels more than the soluble IL2R levels, and viral infection was associated primarily with a marked rise in soluble IL2R. Thus, the combination of tests for serum IL2R and creatinine had greater predictive value than either test alone for distinguishing acute rejection from infection or CsA toxicity. The strength of the combination test is evident in Table XXXIII. If a rise in serum IL2R accompanies a rise in creatinine, the odds are 20:1 in favor of rejection over CsA toxicity. In contrast, if no IL2R rise accompanies the rise in creatinine, the odds favor CsA over rejection by about 3:2 (the majority of patients will be stable).

In these studies and others (Colvin, R.B., et al., 1987, Clin. Immunol. Immunopathol. 43:273-276; Solc, V. and Krause, J.R., 1987, Diag. Clin. Immunol. 5:171-174), infection was accompanied by levels of serum IL2R (up to 33,825 units/ml during a cytomegalovirus infection in this series) even higher than those observed during allograft rejection, suggesting a quantitative or qualitative difference in T cell activation. Accordingly, extremely high levels of IL2R favor the diagnosis of infection over rejection. Finally, transient elevations in serum IL2R are sometimes observed in clinically stable transplant recipients. These have been categorized here as "false positives" but may be caused by subclinical episodes of rejection or infection.

IL2R assays can also be valuable in the monitoring of immunologic activity in recipients of other organ allografts, such as the heart, liver and pancreas, in which the early diagnosis of rejection is particularly difficult.

9. DEPOSIT OF HYBRIDOMAS

The following hybridoma cell lines, producing the indicated monoclonal antibody, have been deposited with the American Type Culture Collection, Rockville, Maryland, and have been assigned the listed accession numbers:

Hybridoma	Monoclonal Antibody	Accession Number
Cell line AM92/2R12	AM92/2R12 (anti-IL2R)	HB 9341
Cell line 7G7	7G7 (anti-IL2R)	HB 8784
Cell line 4C9	4C9 (anti-CD8)	HB 9340
Cell line 5F4/7B12	5F4/7B12 (anti-CD8)	HB 9342
Cell line 8F4	8F4 (anti-CD4)	HB 9843
Cell line R2B7	R2B7 (anti-CD4)	HB 9842

The present invention is not to be limited in scope by the cell lines deposited since the deposited embodiments are intended as single illustrations of one aspect of the invention and any cell lines which are functionally equivalent are within the scope of this invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

International Application No: PCT/

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 74, line 1-25 of the description [1]

A. IDENTIFICATION OF DEPOSIT [2]

Further deposits are identified on an additional sheet [X]

Name of depositary institution [4]

American Type Culture Collection

Address of depositary institution (including postal code and country) [5]

12301 Parklawn Drive
Rockville, MD 20852 U.S.A.

Date of deposit [3]	Accession Number [6]
April 18, 1985	HB 8784

B. ADDITIONAL INDICATIONS [7] (leave blank if not applicable). This information is continued on a separate attached sheet [ ]

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE [8] (if the indications are not for all designated States)

D. SEPARATE FURNISHING OF INDICATIONS [9] (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later [9] (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. [ ] This sheet was received with the international application when filed (to be checked by the receiving Office)

(Authorized Officer)

[ ] The date of receipt (from the applicant) by the International Bureau [10]

was

(Authorized Officer)

Form PCT/RO/134 (January 1981)

Form 134 Cont.

Name of Depository Institution: American Type Culture Collection

Address of Depository Institution: 12301 Parklawn Drive
Rockville, MD 20852 U.S.A.

Date of Deposit	Accession Number
March 3, 1987	HB 9340
March 3, 1987	HB 9341
March 3, 1987	HB 9342
September 28, 1988	HB 9842
September 28, 1988	HB 9843

WHAT IS CLAIMED:

1. A method for detecting or measuring the amount in a sample of a soluble molecule carrying antigenic determinants of the CD4 T cell surface antigen comprising:

(a) contacting the sample with a first anti-CD4 monoclonal antibody and with a second anti-CD4 monoclonal antibody that does not compete for the same binding site on the CD4 antigen as the first anti-CD4 antibody under conditions which allow immunospecific binding; and

(b) detecting whether immunospecific binding occurs of a component in the sample with both first and second anti-CD4 antibodies, in which immunospecific binding of a component of the sample with both first and second anti-CD4 antibodies indicates the presence of the soluble molecule,

in which the sample contains only such soluble molecules as are spontaneously released.

2. The method according to claim 1 in which the second antibody is labeled so that it is capable of producing a detectable signal.

3. The method according to claim 1 in which the first antibody is immobilized.

4. The method according to claim 1 in which the first antibody is immobilized and the second antibody is labeled so that immunospecific binding is indicated by the detection of immobilized label.

5. The method according to claim 1, 2 or 4 in which the first antibody comprises monoclonal antibody 8F4 as produced by the hybridoma deposited with the ATCC and assigned accession number HB 9843.

6. The method according to claim 1, 2 or 4 in which the second antibody comprises monoclonal antibody R2B7 as produced by the hybridoma deposited with the ATCC and assigned accession number HB 9842.

7. The method according to claim 1, 2 or 4 in which the first antibody comprises monoclonal antibody 8F4 as produced by the hybridoma deposited with the ATCC and assigned accession number HB 9843, and the second antibody comprises monoclonal antibody R2B7 as produced by the hybridoma deposited with the ATCC and assigned accession number HB 9842.

8. The method according to claim 1 in which the first antibody has the same epitope specificity as that of monoclonal antibody 8F4 as produced by the hybridoma deposited with the ATCC and assigned accession number HB 9843.

9. The method according to claim 1 in which the second antibody has the same epitope specificity as that of monoclonal antibody R2B7 as produced by the hybridoma deposited with the ATCC and assigned accession number HB 9842.

10. The method according to claim 1 in which the first antibody has the same epitope specificity as that of monoclonal antibody 8F4 as produced by the hybridoma deposited with the ATCC and assigned accession number

HB 9843, and the second antibody has the same epitope specificity as that of monoclonal antibody R2B7 as produced by the hybridoma deposited with the ATCC and assigned accession number HB 9842.

11. The method according to claim 1 or 4 in which the sample is serum.

12. A kit for measuring the level of a cell free soluble molecule carrying antigenic determinants of the CD4 T cell surface antigen comprising:

(a) a first anti-CD4 monoclonal antibody; and

(b) a second anti-CD4 monoclonal antibody that does not compete for the same binding site on the CD4 antigen as the first anti-CD4 antibody.

13. The kit of claim 12 in which the second anti-CD4 antibody is labeled so that it is capable of producing a detectable signal.

14. The kit of claim 13 in which the label is an enzyme.

15. The kit of claim 14 in which the enzyme is horseradish peroxidase.

16. The kit of claim 12 in which the first antibody comprises monoclonal antibody 8F4 as produced by the hybridoma deposited with the ATCC and assigned accession number HB 9843.

17. The kit of claim 12 in which the second antibody comprises monoclonal antibody R2B7 as produced by the hybridoma deposited with the ATCC and assigned accession number HB 9842.

18. The kit of claim 12 in which the first antibody comprises monoclonal antibody 8F4 as produced by the hybridoma deposited with the ATCC and assigned accession number HB 9843, and the second antibody comprises monoclonal antibody R2B7 as produced by the hybridoma deposited with the ATCC and assigned accession number HB 9842.

19. The kit of claim 17 in which monoclonal antibody R2B7 is labeled.

20. The kit of claim 18 in which monoclonal antibody R2B7 is labeled.

21. The kit of claim 20 in which the label is an enzyme.

22. The kit of claim 21 in which the enzyme is horseradish peroxidase.

23. The kit of claim 12 in which the first antibody has the same epitope specificity as that of monoclonal antibody 8F4 as produced by the hybridoma deposited with the ATCC and assigned accession number HB 9843.

24. The kit of claim 12 in which the second antibody has the same epitope specificity as that of monoclonal antibody R2B7 as produced by the hybridoma deposited with the ATCC and assigned accession number HB 9842.

25. The kit of claim 12 in which the first antibody has the same epitope specificity as that of monoclonal antibody 8F4 as produced by the hybridoma deposited with the ATCC and assigned accession number HB 9843, and the second antibody has the same epitope specificity as that of monoclonal antibody R2B7 as produced by the hybridoma deposited with the ATCC and assigned accession number HB 9842.

26. A method for diagnosing a state of immune activation in a subject comprising detecting or measuring an increase, relative to levels in a subject in an unactivated state, in the amount in a sample from the subject of a spontaneously released, soluble molecule carrying antigenic determinants of the CD4 T cell surface antigen.

27. A method for diagnosing a state of immune activation in a subject comprising detecting or measuring an increase, relative to levels in a subject in an unactivated state, in the amount in a sample from the subject of a soluble molecule carrying antigenic determinants of the CD4 T cell surface antigen, according to the method of claim 1.

28. A method for diagnosing a state of immune activation in a subject comprising detecting or measuring an increase, relative to levels in a subject in an unactivated state, in the amount in a sample from the subject of a soluble molecule carrying antigenic determinants of the CD4 T cell surface antigen, according to the method of claim 7.

29. A method for determining the phenotype of a T cell comprising incubating the cell in vitro, and detecting or measuring the amount, in a sample of the cell culture

fluid, of a spontaneously released, soluble molecule carrying antigenic determinants of the CD4 T cell surface antigen.

30. A method for staging adult T cell leukemia in a patient comprising measuring the amount of a spontaneously released, soluble molecule carrying antigenic determinants of the CD4 T cell surface antigen in a sample obtained from the patient, in which increases in amounts relative to healthy individuals or to the patient at an earlier time indicates more advanced stages of disease.

31. The method according to claim 30 in which the sample is serum.

32. A method for diagnosing rheumatoid arthritis in a patient comprising detecting or measuring an increase in the amount, relative to healthy individuals or to the patient at an earlier time, of a spontaneously released, soluble molecule carrying antigenic determinants of the CD4 T cell surface antigen in synovial fluid obtained from the patient.

33. A method for diagnosing rheumatoid arthritis in a patient comprising detecting an increase in the amount in synovial fluid relative to the amount in serum of a spontaneously released, soluble molecule carrying antigenic determinants of the CD4 T cell surface antigen.

34. A method for monitoring the treatment of a patient with a viral infection comprising measuring the level in a body fluid of the patient of a spontaneously released, soluble molecule carrying antigenic determinants of the CD4 T cell surface antigen.

35. The method according to claim 34 in which the viral infection is Human Immunodeficiency Virus infection.

36. A monoclonal antibody which preferentially binds to a spontaneously released, soluble molecule carrying antigenic determinants of the CD4 T cell surface antigen, relative to the CD4 T cell surface antigen.

37. A rat monoclonal antibody reactive with (a) a spontaneously released, soluble molecule carrying antigenic determinants of the CD4 T cell surface antigen, and (b) the CD4 T cell surface antigen.

38. Monoclonal antibody 8F4, as produced by the hybridoma deposited with the ATCC and assigned accession number HB 9843.

39. Monoclonal antibody R2B7, as produced by the hybridoma deposited with the ATCC and assigned accession number HB 9842.

40. The Fv, Fab, Fab', or $F(ab')_2$ fragment of the monoclonal antibody of claim 36, 37, 38 or 39.

41. An antibody comprising the Fv, Fab, Fab', or $F(ab')_2$ fragment of the monoclonal antibody of claim 36, 37, 38 or 39.

42. A method for differentially diagnosing allograft rejection from infection in a transplant patient comprising:

(a) measuring the amount of a spontaneously released, soluble molecule carrying antigenic determinants of the

interleukin-2 receptor in a sample obtained from the transplant patient; and

(b) measuring the amount of creatinine in a body fluid obtained from the patient,

in which increases in both the amount of the molecule and in the amount of creatinine, relative to healthy subjects without a transplant or not undergoing rejection or to the patient at an earlier time, indicates rejection of the allograft.

43. The method according to claim 42 in which the sample comprises serum, and the body fluid comprises serum.

44. The method according to claim 42 in which the sample comprises urine, and the body fluid comprises urine.

45. The method according to claim 42 in which the soluble molecule carrying antigenic determinants of the interleukin-2 receptor is detected by:

(a) contacting the sample with monoclonal antibody 2R12, as produced by the hybridoma deposited with the ATCC and assigned accession number HB 9341, and with monoclonal antibody 7G7, as produced by the hybridoma deposited with the ATCC and assigned accession number HB 8784, under conditions which allow immunospecific binding; and

(b) detecting whether immunospecific binding occurs of a component in the sample with both monoclonal antibodies 2R12 and 7G7,

in which such immunospecific binding indicates the presence of the soluble molecule in the sample.

46. The method according to claim 42 in which the allograft comprises a renal transplant.

47. The method according to claim 42 in which the allograft comprises a cardiac transplant.

48. A method for detecting, monitoring the treatment of, or staging a disease or disorder in a patient comprising measuring the levels in a body fluid of the patient of a plurality of spontaneously released, soluble molecules, which molecules are selected from the group consisting of molecules carrying antigenic determinants of the interleukin-2 receptor, molecules carrying antigenic determinants of the CD4 antigen, and molecules carrying antigenic determinants of the CD8 antigen.

49. The method according to claim 48 which further comprises measuring the changes in the levels of the molecules relative to the levels in healthy individuals or in the patient at an earlier time.

50. The method according to claim 48 in which the disease or disorder comprises transplant rejection.

51. The method according to claim 49 in which the disease or disorder comprises transplant rejection.

52. The method according to claim 50 in which the transplant is a renal transplant.

53. The method according to claim 52 in which renal transplant rejection is differentially diagnosed from cyclosporin A toxicity.

54. The method according to claim 48 in which the disease or disorder is caused by viral infection.

55. The method according to claim 49 in which the disease or disorder is caused by viral infection.

56. The method according to claim 54 or 55 in which the viral infection is Human Immunodeficiency Virus infection.

57. The method according to claim 48 in which the disease or disorder comprises rheumatoid arthritis.

58. The method according to claim 49 in which the disease or disorder comprises rheumatoid arthritis.

59. A kit for detecting, monitoring the treatment of, or staging a disease or disorder in a patient comprising:

(a) a pair of anti-interleukin-2 receptor monoclonal antibodies, which antibodies do not compete for the same binding site on the interleukin-2 receptor; and

(b) a pair of anti-CD4 monoclonal antibodies, which antibodies do not compete for the same binding site on the CD4 antigen.

60. A kit for detecting, monitoring the treatment of, or staging a disease or disorder in a patient comprising:

(a) a pair of anti-interleukin-2 receptor monoclonal antibodies, which antibodies do not compete for the same binding site on the interleukin-2 receptor; and

(b) a pair of anti-CD8 monoclonal antibodies, which antibodies do not compete for the same binding site on the CD8 antigen.

61. A kit for detecting, monitoring the treatment of, or staging a disease or disorder in a patient comprising:

(a) a pair of anti-CD8 monoclonal antibodies, which antibodies do not compete for the same binding site on the CD8 antigen; and

(b) a pair of anti-CD4 monoclonal antibodies, which antibodies do not compete for the same binding site on the CD4 antigen.

62. The kit of claim 59 which further comprises a pair of anti-CD8 monoclonal antibodies, which antibodies do not compete for the same binding site on the CD8 antigen.

FIG. 1

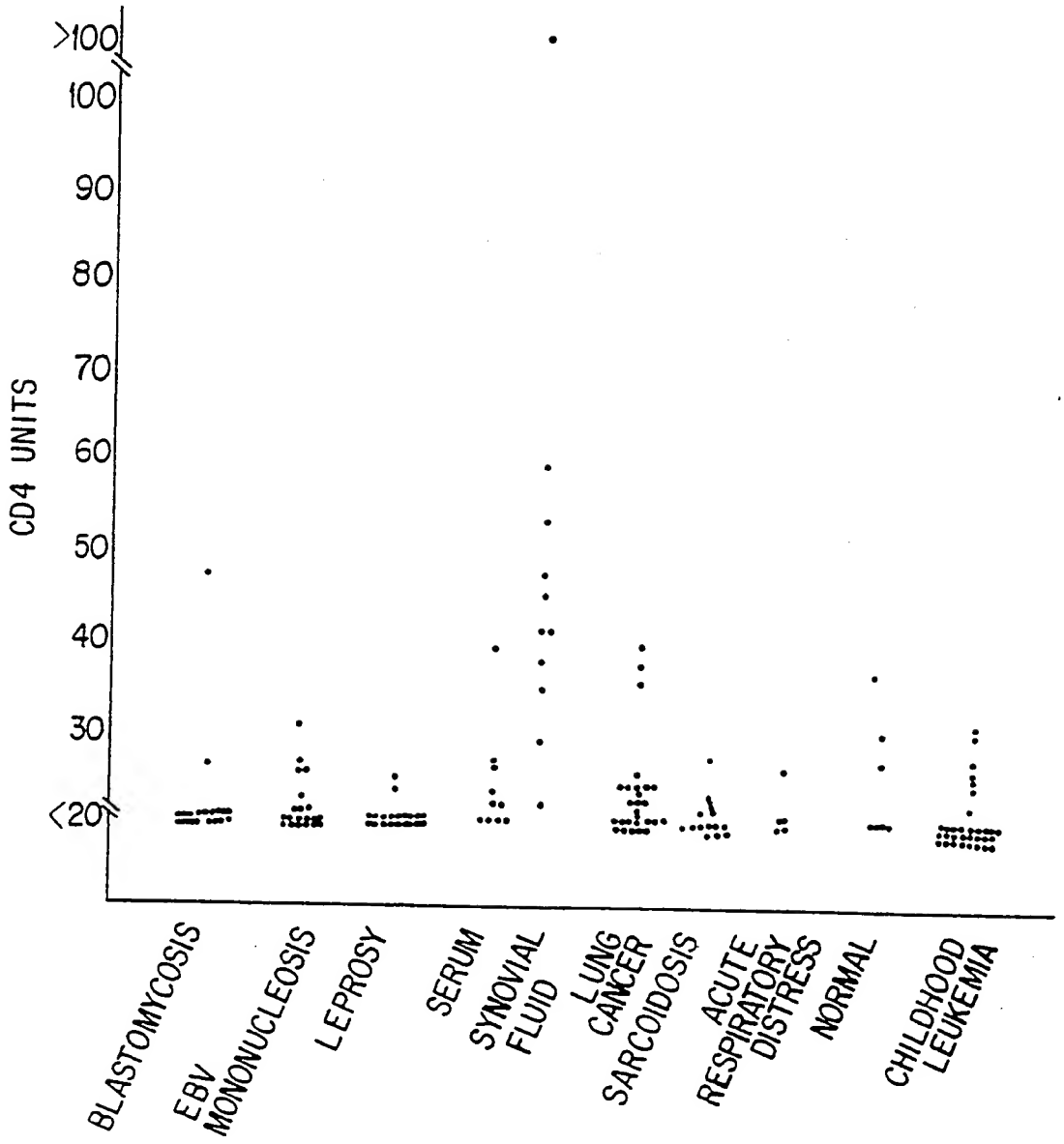

>100
100
90
80
70
60
50
40
30
<20
CD4 UNITS
BLASTOMYCOSIS
EBV MONONUCLEOSIS
LEPROSY
SERUM
SYNOVIAL FLUID
LUNG CANCER
SARCOIDOSIS
ACUTE RESPIRATORY DISTRESS
NORMAL
CHILDHOOD LEUKEMIA

FIG. 2

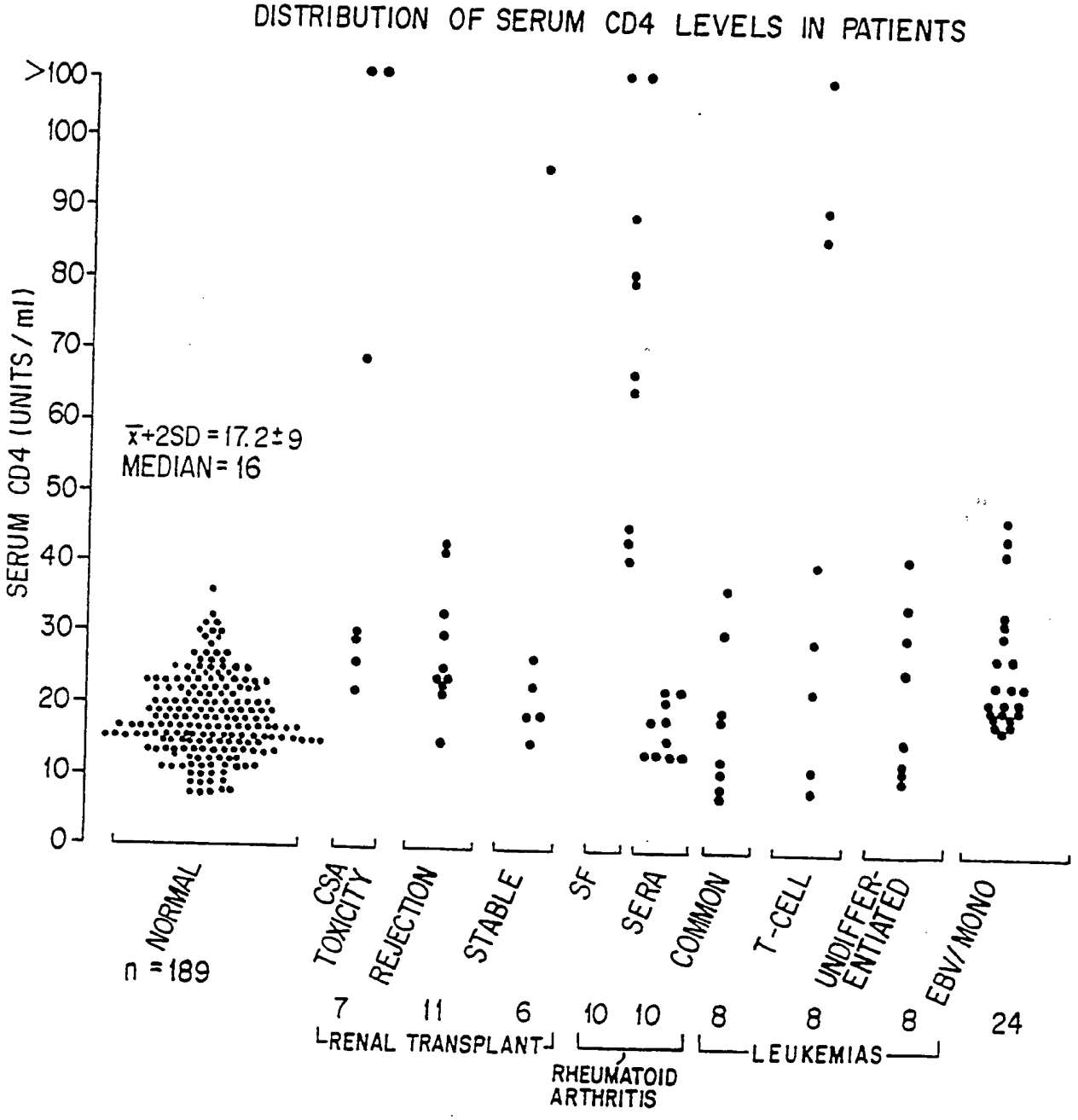

DISTRIBUTION OF SERUM CD4 LEVELS IN PATIENTS
SERUM CD4 (UNITS / ml)
>100
100
90
80
70
60
50
40
30
20
10
0
x̄+2SD = 17.2±9
MEDIAN = 16
NORMAL
n = 189
CSA TOXICITY
REJECTION
STABLE
SF
SERA
COMMON
T-CELL
UNDIFFER-ENTIATED
EBV/MONO
7
11
6
10
10
8
8
8
24
RENAL TRANSPLANT
RHEUMATOID ARTHRITIS
LEUKEMIAS

FIG.3A

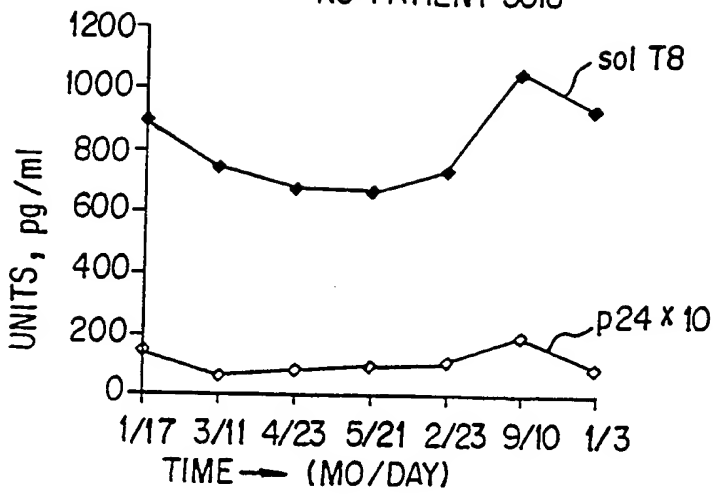


FIG.3B

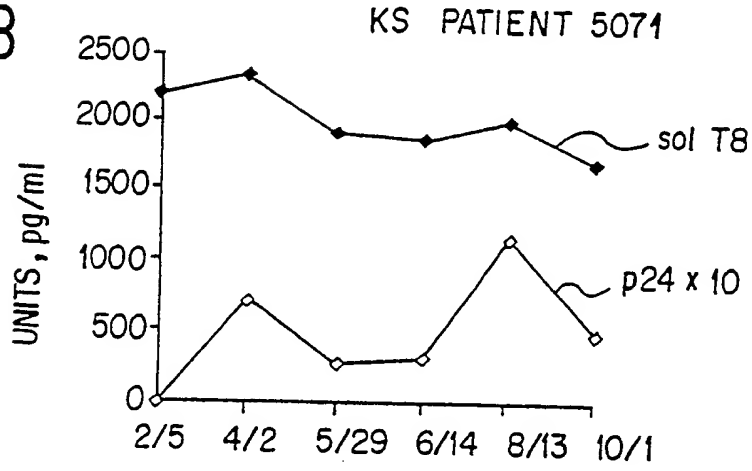


FIG.3C

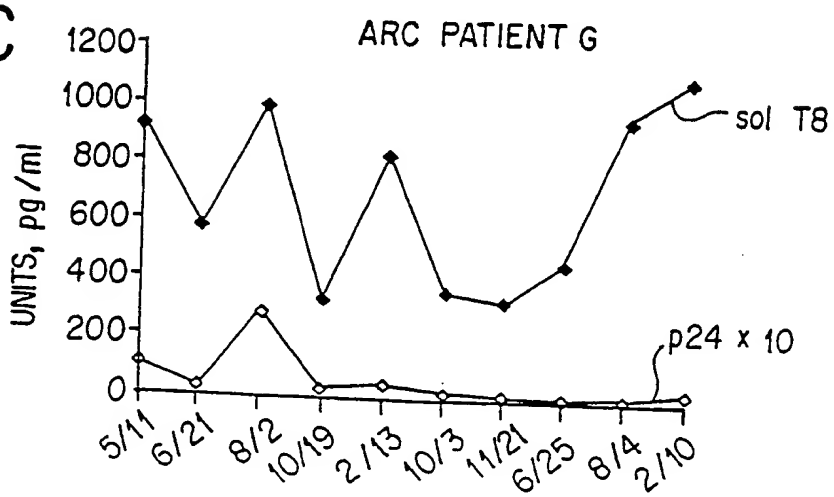


FIG. 4A

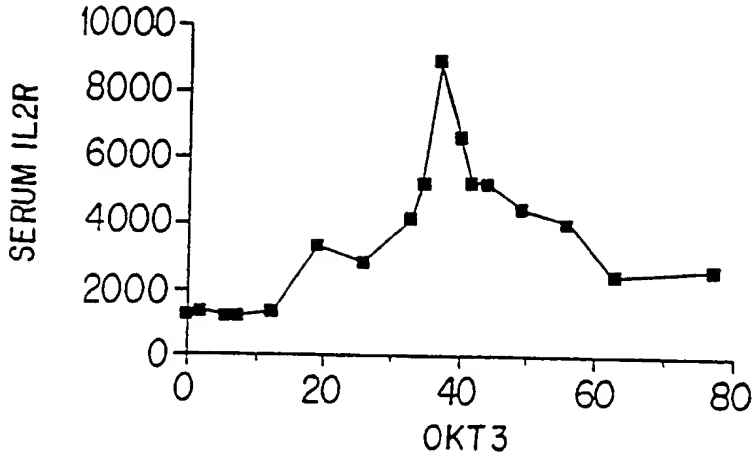

SERUM IL2R
10000
8000
6000
4000
2000
0
0
20
40
60
80
OKT3

FIG. 4B

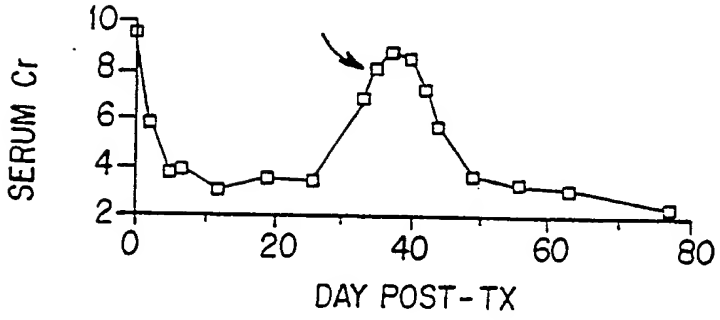

SERUM Cr
10
8
6
4
2
0
20
40
60
80
DAY POST-TX

FIG.5A

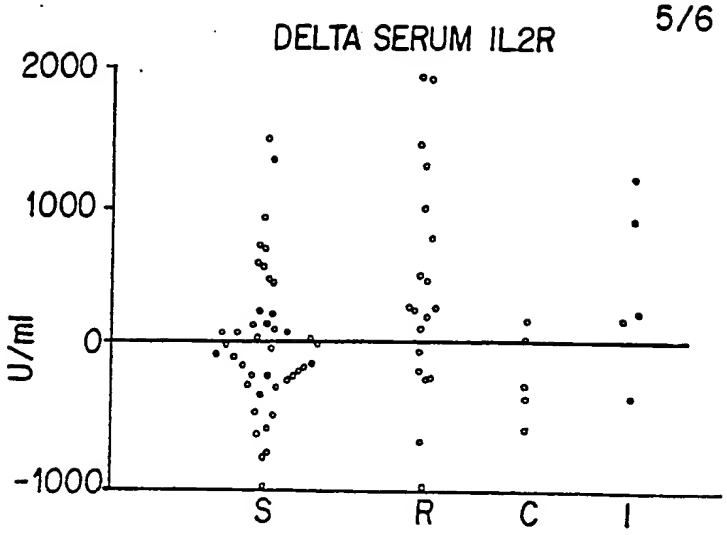


FIG.5B

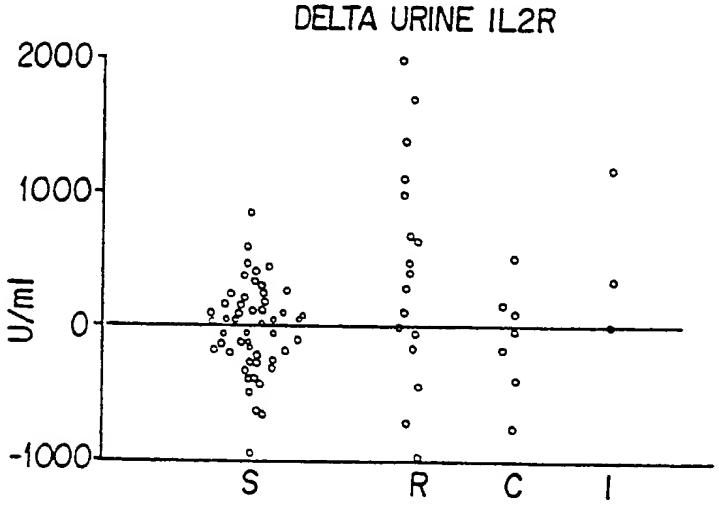


FIG.5C

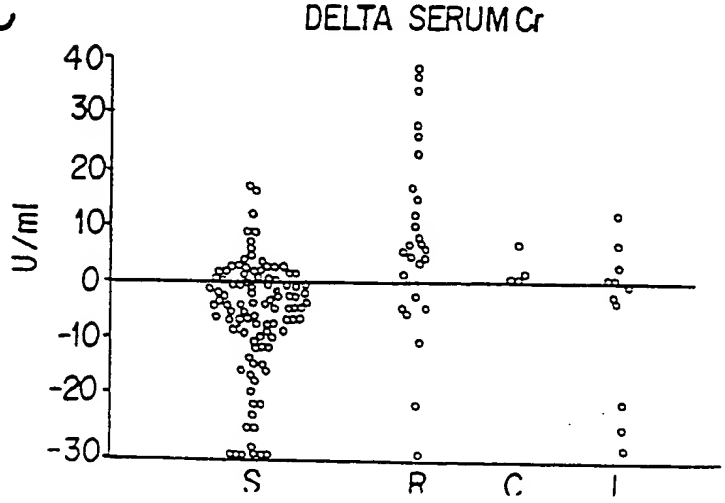
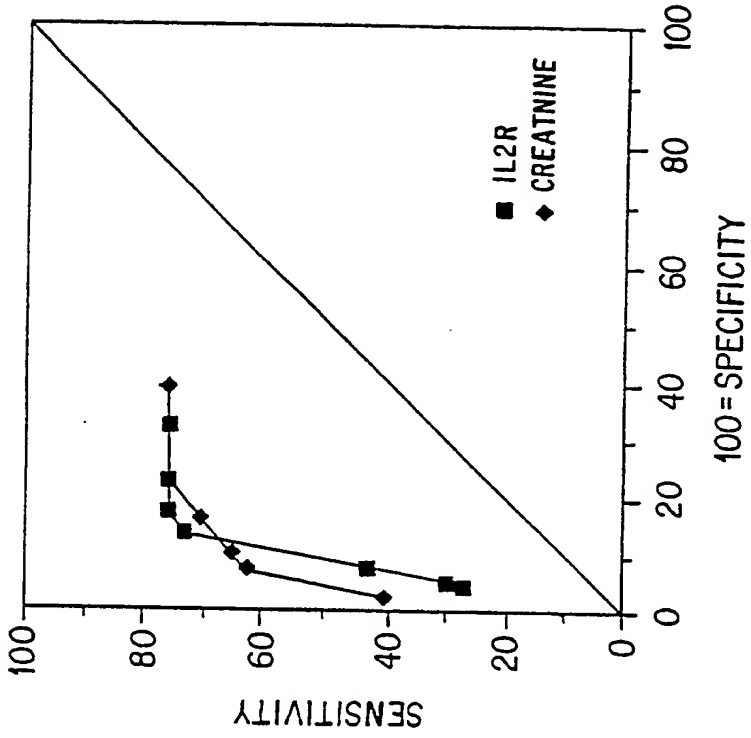


FIG. 6


IL2R
CREATNINE
SENSITIVITY
100=SPECIFICITY
100
80
60
40
20
0
100
80
60
40
20
0

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US89/04413

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

US. CL: 435/5, 7, 34, 810; (See Attachment).

II FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
US. CL:	435/5,7,34,810; 436/501,503,506, 510,518,536,548,811,813; 530/387,391,809; 935/110

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

CAS and Biosis Searches:
((Release? or Shed? or Solub?) (2A) (CD4 or T4))/BI, AB

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	US,A, 4,707,443 (NELSON ET AL) 17 November 1987. See col. 13, lines 20-33.	42-56 and 60
X	WO, A, 8705912 (KUNG ET AL) 8 October 1987. See pages 46-48, claims and Search Report.	42-56 and 60
Y	DT, A, 3628718 (BAYER AG) 4 June 1987. See abstract and page 2, last full paragraph.	42-56 and 60
X / Y	Biological Abstracts, Vol. 86, no. 1, issued 1 July 1988. (Philadelphia, PA, USA), E.A. Berger et al. "A soluble recombinant polypeptide comprising the amino terminal half of the extracellular region of the CD4 molecule contains an active binding site for human immunodeficiency virus".	36,37 / 40,41

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search

06 November 1989

Date of Mailing of this International Search Report

21 DEC 1989

International Searching Authority

ISA/US

Signature of Authorized Officer

David A. Saunders

David A. Saunders

Form PCT/ISA/210 (second sheet) (Rev.11-87)

Attachment to Form PCT/ISA/210
Part I. Classification Of Subject Matter

US CL: 436/501,503,506,510,518,536,548,811,813;
530/387,391,809;935/110

IPC 4: G01N 33/535,
543,564,567,571,574,577

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

X Y	Jour. Exp. Med., vol. 59, no. 3, issued September 1983, J. FUJIMOTO ET AL, "Spontaneous release of the leu-2 (T8) molecule from human T-cells", pages 752-765. See pages 753, 757 and 760.	1-4, 11-15, 48 and 49 60-62

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers . because they relate to subject matter [2] not required to be searched by this Authority, namely:

2. ☐ Claim numbers ..... , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [3], specifically:

3. ☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [3]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.